IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. No.	:	09/973,089	Confirmation No. 4802
Applicant	:	Marie B. CONNETT-PORCEDDU	
Filed	:	10 October 2001	
TC/A.U.	:	1638	
Examiner	:	Stuart F. Baum	
Docket No.	:	2411-111	
Customer No.	:	6449	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER RULE 132 OF MICHAEL R. BECWAR

Dear Sir:

I, Michael R. Becwar, declare as follows:

1. My education and experience are as follows. I received a Bachelor of Science degree in Physics and Mathematics from University of Wisconsin in 1970, a Masters of Science degree in Horticulture from Oregon State University in 1976 and a Doctorate degree in Plant Physiology from Colorado State University in 1980. I have been employed by Arborgen, LLC, which is a joint venture including Westvaco Corporation, the assignee of the present application, from 2000 to present as a Science Fellow. I held the position of Research Scientist with Westvaco Corporation from 1988 to 1999. I was employed by Institute of Paper Science and Technology from 1984 to 1988, as an Industrial Research Fellow. I started the research and development program on pine somatic embryogenesis at Westvaco Corporation. My research emphasis is with somatic embryogenesis for clonal propagation and genetic engineering of conifers. I have been involved with tissue culture of pines since 1988.

2. My own research, as well as my professional interests and background have given me a good knowledge and understanding of conifer transformation and in particular the cell and tissue culture methods and systems that are an integral part of developing conifer transformation systems. Accordingly, I believe that I am well qualified to render a professional opinion regarding the positions set forth in the Offices Action and the nonobviousness of the invention described in the above application.

3. I understand that the claims recite a method for regenerating genetically modified plants of pine of the genus *Pinus* selected from the group consisting of Southern yellow pines and hybrids thereof. This method provides for enhanced regeneration of transgenic embryogenic pine cells for this important group of pines.

4. I have recently reviewed this application and the Office Action mailed January 28, 2003. I have also recently reviewed the Wenck et al. (*Plant Mol Biol* 39:407-416, 1999), Levee et al. (*Molecular Breeding* 5:429-440, 1999) and Rutter et al. (US 5,731,204) references cited in the Office Action.

5. I understand that the Examiner has stated that the claimed invention is unpatentable because it is anticipated by Wenck et al. because Wenck et al. "use Phytagel, which is a gelling agent and the 471 medium which they use comprises nutrients which act as an osmoticum." Office Action at page 4.

6. I also understand that the Examiner has stated that the claimed invention is unpatentable over Wenck et al. and Rutter et al. because it would have been obvious to persons skilled in the art to use the abscisic acid (ABA) and polyethylene gycol (PEG) of Rutter et al. in the selection medium of Wenck et al. to enhance the efficiency of transformation and regeneration of pine plants.

7. I further understand that the Examiner has stated that the claimed invention is unpatentable over Wenck et al and Levee et al. because it would have been obvious to persons skilled in the art to use the support membrane of Levee et al. in the method of Wenck et al.

8. I also understand that the Examiner has stated that the claimed invention is unpatentable over Wenck et al., Levee et al. and Rutter et al. because it would have been obvious to persons skilled in the art to use the support membrane of Levee et al. and the ABA of Rutter et al. in the method of Wenck et al.

9. Applicants have invented a method which provides enhanced regeneration of transgenic embryogenic pine cells in which the pine is a Southern yellow pine or a hybrid thereof. These pines belong to the genus *Pinus*, subgenus *Pinus*. Pines of the subgenus *Pinus* are hard pines. See Little and Critchfield, 1969, *Subdivision of the genus Pinus (Pines)*, USDA Forest Service Miscellaneous Publication 1144, Washington, D.C. (attached as Exhibit 1 to Declaration Under Rule 132 of Dr. Connett-Porceddu, filed concurrently herewith). The method involves selecting

transgenic embryogenic pine cells using a selection medium that contains a selection agent and an agent that regulates differentiation. The differentiation regulating agent may be ABA, PEG, a gelling agent in an amount of 0.5%-1.5% or an amount of 3%-5%, or mixtures thereof. Applicants discovered that, through the use of the disclosed and claimed method, they were able to select and regenerate transgenic pine plants of Southern yellow pines and hybrids thereof. These pines belong to the *Pinus* subgenus, i.e. hard pines. Applicants' invention allowed for the first time the efficient regeneration of transgenic plants of these economically important pines at significant frequency from transgenic pine cells.

10. I have worked with both eastern white pine (*Pinus strobus*) and hard pines (including *Pinus taeda*). In fact, my group was the first to publish obtaining somatic embryogenic cultures from *P. strobus* (Becwar et al. 1988. In: Ahuja, MR (ed) *Somatic cell genetics of woody plants*, Kluwer Academic Publ., Dordrecht, The Netherlands. pp. 1-13; copy attached as Exhibit 1). Although there are similarities in the stage for culture initiation and the general appearance of embryogenic cultures of *P. strobus* and hard pines such as *P. taeda*, the similarities end there. It is generally known and well accepted by those skilled in conifer somatic embryogenesis that what works with one group of pines will not necessarily work with another group of pines. For example, success in somatic embryogenesis with *Pinus strobus*, for example obtaining high culture initiation rates of 54% as reported by Finer et al. (1989. Initiation of embryogenic callus and suspension cultures of eastern white pine (Pinus strobus L.). *Plant Cell Rep.* **8**:203-206; copy attached as Exhibit 2), does not assure or convey success either within the same species or with other *Pinus* species. Even though the Finer et al. report appeared in the late 80's it was not until 2001 that researchers reported the first real measure of broad success in somatic embryogenesis with *P. strobus* (Klimaszewska et al. 2001. Optimized somatic embryogenesis in Pinus strobus L. *In Vitro Cell. Dev. Biol. Plant* **37**:392-399; copy attached as Exhibit 3). Additional facts supporting this knowledge and lack of success between soft and hard pines can be found in the Declaration Under Rule 132 of Dr. Connett-Porceddu in companion application Serial No. 09/973,088 (copy attached as Exhibit 2 to the Declaration Under Rule 132 of Dr. Connett-Porceddu filed concurrently herewith). Indeed, the knowledge and acceptance in the art that what works with one group of pines will not necessarily work with another group of pines has been my experience in my work with soft and hard pines.

11. As shown in the present application, genetically modified plants are obtained using the claimed method in which transgenic embryogenic pine cells of Southern yellow pines or hybrids thereof are selected on a medium containing a selection agent and the specified agents that regulate differentiation, i.e., ABA, PEG, gelling agent in the specified amount or mixtures thereof. Wenck et al., on the other hand, teaches that genetically modified plants had not been obtained. Specifically, Wenck et al. at page 413, bottom of left column with respect to loblolly pine, a Southern yellow pine, states: "[w]e have not been able to recover stable transformants through selection to date." Thus, the method described in Wenck et al. did not produce genetically modified plants of Southern yellow pines or hybrids thereof.

12. Furthermore, in my opinion, the report by Wenck et al. would not make obvious the methods taught in the invention in the present application. Wenck et al. only obtained transient transformation in loblolly pine. That is, they did not teach a method that will result in obtaining stably transformed (transgenic) loblolly pine trees. Thus, the work was primarily with Norway spruce and very little was done with loblolly pine. It was a scientific exaggeration for the authors to include in the title "high efficiency" transformation with both species. It would have been more correct to title the work "Improvement in the expression of GUS marker gene in Norway spruce," and to not include loblolly pine in the title since so little was done with that species. In contrast, the invention of described in the present application specifically teaches methods to obtain stably transformed transgenic loblolly pine trees. Furthermore, the invention described in the present application provides methods that have commercial-scale applicability. This in itself separates the present invention from the published literature, including that by Wenck et al.

13. Rutter et al. describes a method for regenerating pine plants from tissue culture. This method involves the use of PEG and ABA during a very distinct and specific stage of somatic embryogenesis, namely during the stage when cultures are induced to develop somatic embryos and for the embryos to mature. This stage is sometimes referred to as "embryo maturation" since it is during this step that the cultures undergo a morphological change from having relatively undifferentiated tissue with only very early stage embryos to a culture having more fully developed (mature) embryos. This process is completely different than the use of ABA and/or PEG during the selection process for obtaining stable transgenic cell lines as disclosed in the present application. The physiological reason ABA and/or PEG is applied in the embryo maturation case (Rutter et al.) is

to induce the very early stage embryos to develop and mature. During the selection process of the present invention, ABA and/or PEG is not being used to induce development or maturation of the embryos. Rather, the ABA and/or PEG appears to stimulate the growth of the transgenic tissue. These two responses are different. There is nothing in Rutter et al. which suggests that ABA and/or PEG should be used in the selection process. Furthermore, Rutter et al. is directed to methods for somatic embryogenesis and is not directed to methods for obtaining transgenic plants, nor are transgenic plants obtained in Rutter et al. Since Wenck et al. did not regenerate genetically modified plants from selected transgenic pine cells and Rutter et al. does not select transgenic pine cells and does not regenerate plants from selected transgenic pine cells, it is my opinion that a skilled artisan would have no expectation of success for modifying the method of Wenck et al. as proposed by the Examiner to regenerate genetically modified plants of Southern yellow pines or hybrids thereof.

14. The Examiner cites Levee et al. for the use of a support membrane. Since Wenck et al. did not regenerate genetically modified plants from selected transgenic pine cells and Levee et al. does not select transgenic pine cells of Southern yellow pines or hybrids thereof and does not regenerate plants from the selected transgenic pine cells, it is my opinion that a skilled artisan would have no expectation of success for modifying the method of Wenck et al. to regenerate genetically modified plants of Southern yellow pines or hybrids thereof.

15. Since Wenck et al. did not regenerate genetically modified plants from selected transgenic pine cells, Levee et al. does not select transgenic pine cells of Southern yellow pines or hybrids thereof and does not regenerate plants from the selected transgenic pine cells and Rutter et al. does not select transgenic pine cells and does not regenerate plants from selected transgenic pine cells, it is my opinion that a skilled artisan would have no expectation of success for modifying the method of Wenck et al. to regenerate genetically modified plants of Southern yellow pines or hybrids thereof.

16. In summary, Wenck et al. does not teach the regeneration of genetically modified Southern yellow pines or hybrids thereof. Rutter et al. does not teach (a) the selection of transgenic cells of Southern yellow pines or hybrids thereof and (b) the regeneration of genetically modified plants from such cells. Levee et al. does not teach the regeneration of transgenic hard pines, such as Southern yellow pines and hybrids. Thus, there was no expectation of success for modifying the

method of Wenck et al. to regenerate genetically modified pine plants from selected transgenic cells of Southern yellow pines or hybrids thereof.

17. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or of any patent issued thereon.

Nov. 24, 2003
Date

M. R. Becwar
Michael R. Becwar

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DEVELOPMENT AND CHARACTERIZATION OF IN VITRO EMBRYOGENIC SYSTEMS IN CONIFERS

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1. ABSTRACT

Our progress is reviewed on development of somatic embryogenesis in conifers for mass propagation. A distinct embryogenic callus (EC) phenotype, white, mucilaginous, and rapidly growing, has been initiated on modified MS media with 2,4-D or NAA (2-5 mg/L) and BA(0-1 mg/L) from immature embryos of Norway spruce (Picea abies), white spruce (Picea glauca), loblolly pine (Pinus taeda), pond pine (Pinus serotina), and white pine (Pinus strobus). EC has also been initiated from mature embryos of Norway spruce and maintained as rapidly growing (48 hour doubling) liquid suspensions. Initiation of EC in Picea and Pinus differ markedly in several ways. Precotyledonary embryos were optimal in Pinus and EC originated from the suspensor region. In Picea EC originated from the hypocotyl and cotyledon region of predominantly post-cotyledonary embryos. Biochemically, EC of Picea and Pinus were similar and distinctly different from nonembryogenic callus (NEC) in terms of ethylene evolution rates (EC low and NEC high), level of total reductants, including glutathione (EC low and NEC high), and protein synthesis rates (EC high and NEC low). Conifer somatic embryos contained proplastids closely resembling those found in early zygotic embryos. On proliferation medium in the light, EC was white and maintained the proplastid morphology, whereas, NEC was green and contained mature chloroplasts with grana. These biochemical and ultrastructural differences served to both verify and predict embryogenic potential.

With Norway spruce somatic embryos, maturation frequencies as high as 25% have been attained. Germination frequencies as high as 82% (mean 56%) have been obtained. Twenty-nine percent of the somatic embryo plantlets survived transfer to the greenhouse, set a dormant terminal bud, overwintered to -5°C, and renewed vegetative growth synchronously with control seedlings. This is the first report of overwintering and renewed vegetative growth from resting buds of conifer somatic embryo plants.

2. INTRODUCTION

The long life cycle of conifers slows genetic improvement via the traditional sexual breeding process. Clonal (vegetative) propagation techniques show considerable promise for achieving more rapid tree improvement and increased productivity (7,15,20,29). There are three distinct methods of clonal propagation that are applicable to forest trees: macropropagation, micropropagation, and somatic embryogenesis. Macropropagation, the rooting of stem cuttings, is widely used in certain hardwoods as Eucalyptus spp. and softwoods as Norway spruce (Picea abies). Micropropagation involves regeneration from small pieces of tissue from either preexisting meristems, e.g., axillary buds, or from adventitious buds. Although micropropagation has proven utility in the clonal propagation of several commercially important forest trees, including Eucalyptus spp. and radiata pine (Pinus radiata), its primary limitation for mass propagation is the high produc-

M. R. Ahuja (ed.), Somatic Cell Genetics of Woody Plants, 1–18.

Exhibit 1
Becwar

tion cost of individual propagules. Recent advances in regeneration via adventitious bud culture suggest that the technique may be economical for radiata pine (1).

A more promising clonal propagation technique for the economical production of large numbers of propagules is somatic embryogenesis, the production of embryolike structures from somatic tissue under in vitro conditions. Somatic embryos can be produced from cells, thus making highly efficient liquid cell culture techniques available for maintenance and production purposes. Furthermore, somatic embryos can be encapsulated to form artificial seeds for highly efficient delivery to existing tree nursery programs (27).

Somatic embryogenesis has been reported for several coniferous species, including Norway spruce (16), European larch (Larix decidua) (24), radiata pine (28), sugar pine (Pinus lambertiana) (13), loblolly pine (Pinus taeda) (14), and white and black spruce (Picea glauca and P. mariana) (19). In our laboratory we have initiated embryogenic cultures of Norway spruce (3), white spruce (25), loblolly pine, pond pine (Pinus serotina), and white pine (Pinus strobus) (5,35).

Here we review our progress on development and characterization of in vitro embryogenic systems in conifers. Emphasis is placed on similarities and differences between embryogenic systems in Picea and Pinus.

3. INITITATION OF EMBRYOGENIC CALLUS IN CONIFERS

3.1 Picea

3.1.1. Origin of embryogenic callus. Immature embryos of Norway spruce and white spruce produce both an embryogenic callus (EC) and a nonembryogenic callus (NEC) when cultured on basal medium supplemented with auxin and cytokinin (16,19). Recent investigations in our laboratory have provided detailed information on the nature and site of origin of the EC (25). Immature embryos of Picea cultured on callus induction medium produced two types of white to translucent calli that were phenotypically distinct. The callus that proliferated from the hypocotyl region was white to translucent, glossy, mucilaginous, and embryogenic. The epidermal and subepidermal tissue of the hypocotyl gave rise to the mucilaginous callus. After about 10 days in culture this callus gave rise to early stage somatic embryos consisting of an embryonal initial and suspensor initial cells. These results showed that in Picea a callus phase preceded somatic embryogenesis. The other type of white callus originated from the radicle and was nonembryogenic. Other investigations (19) have provided corroborative evidence for the hypocotyl origin of EC in Picea.

Numerous immature and mature embryos of Picea have been cultured on a variety of callus-inducing media, and initiation of EC from the radical region as reported by Gupta and Durzan (12) was not observed. We have observed initiation of EC at very low frequencies from cotyledons of 10-14 day old germinants of Norway spruce. This was reported earlier by Krogstrup (22). Recently, Lelu (23) initiated EC from 23% of cotyledons of 3-7 day old germinants of Norway spruce.

3.1.2. Optimum initiation window. In Norway spruce EC was initiated from immature embyros on the basal medium of von Arnold and Eriksson (31) supplemented with 2,4-D and BA (2 and 1 mg/L, respectively). All Picea cultures were initiated and maintained at 23°C with 16 hr irradiance (15-50 $\mu E \cdot m^{-2} \cdot sec^{-1}$) from cool-white fluorescent and incandescent lights. Initiation frequencies as high as 75% were attained from embryos collected in Wisconsin during July, 1985 (3). EC was also initiated from a different source tree during the summer of 1986 and the results are summarized in

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Table 1. During the four week period from June 30 to July 21, initiation of EC was significantly higher than earlier or later collections. During the period of high initiation, embryo explants were predominantly cotyledonary. A 14-day cold (4°C) storage period prior to culture initiation did not significantly increase initiation (Table 1). Hakman and von Arnold (17) reported that a cold pretreatment of cones for two months increased EC initiation. The 14-day cold pretreatment period we used may not have been long enough to significantly increase initiation. Regardless, these results demonstrate the utility of cold storage for extending the time available for initiation from immature embryos of Picea.

TABLE 1. Frequency of initiation of EC from immature embryo explants of freshly collected and cold stored cones of Norway spruce.

Collection time month/date	Explant length (mm)	Explants with cotyledons, (%)	Embryogenic callus initiation (%) from explants[1]	
			fresh[2]	cold stored
6/23	0.2 ± 0.1	0	6 a	22 a
6/30	0.5 ± 0.1	0	59 b	34 b
7/7	0.9 ± 0.4	64	44 b	6 a
7/14	2.3 ± 0.4	83	62 b	72 b
7/21	3.4 ± 0.2	100	55 b	65 b
8/4	—	100	0 a	--

[1]Twenty-five explants per each treatment, 5 explants per plate. Data are mean values among plates. Means within rows followed by the same letter are not significantly different ($p = 0.05$).

[2]Means followed by the same letter are not significantly different.

The optimum window for initiation of EC from immature embryos of white spruce has also been determined (Figure 1). During the summer of 1986 a total of 388 immature embryo explants of white spruce were cultured on a modified von Arnold and Eriksson (31) basal medium supplemented with 2,4-D and BA (2 and 1 mg/L, respectively). Culture conditions were identical to those used for initiation from immature Norway spruce embryos (3). Cotyledonary embryos of about 1.5 to 2.0 mm were the most effective explants for initiation of EC. Thus, embryo length and the stage of development of cotyledonary primordia were useful indices for identifying the optimum stage for initiating EC from white spruce immature embryos.

The window for initiation of EC in Norway spruce has been extended to mature enbryos by utilizing a modified basal medium, BLG. This medium as described by Amerson (2), is a modified MS medium. The modifications include replacement of NH_4NO_3 with 10 mM glutamine; reduction of KNO_3 from 1900 to 100 mg/L, $MgSO_4$ from 370 to 320 mg/L, sucrose from 3 to 2%; and the addition of 100 mg/L asparagine and 745 mg/L of KCl. The results are fully summarized elsewhere (6). In brief, the results showed that by culturing mature embryos in the light (16 hr photoperiod) on half-strength BLG with either 2,4-D or NAA and BA, approximately 25% of the explants intitiated EC and a majority of the lines were successfully maintained. In contrast, initiation of EC from mature embryos was achieved at a very low frequency (< 3%) on the protocol used for immature embryos, and none of these lines could be maintained. Furthermore, the change to full-strength BLG did not result in initiation of EC from mature embyros. These results point out the importance of the levels and interactions of several components of the

BLG medium for extending the initiation of EC to mature embryos. Von Arnold and Hakman (32) have been able to initiate EC from mature embryos of Norway spruce cultured in the dark on the von Arnold and Eriksson (31) medium by reducing the sucrose level from 3.4 to 1%. Taken collectively, our results on initiation from mature embryos and those of von Arnold (30) demonstrate that changes in and optimization of medium components can significantly affect initiation and therefore play a major role in extending the initiation window to more mature tissues.

3.1.3. Quantification of embryogenic capacity. A method for quantitative determination of the level of somatic embryogenesis in conifer EC has been developed (4). EC of Norway spruce was dispersed in liquid by agitation and plated in a thin layer of medium containing 0.6% low melting point agarose. The density of somatic embryos ranged from 200 to 1500 per gram of EC among 11 lines surveyed. Thus, the somatic embryo counting technique was useful for identifying highly embryogenic lines among those with similar phenotypes. This technique has also proven useful for evaluating the effectiveness of biochemical treatments aimed at enhancing the level of embryogenesis (33).

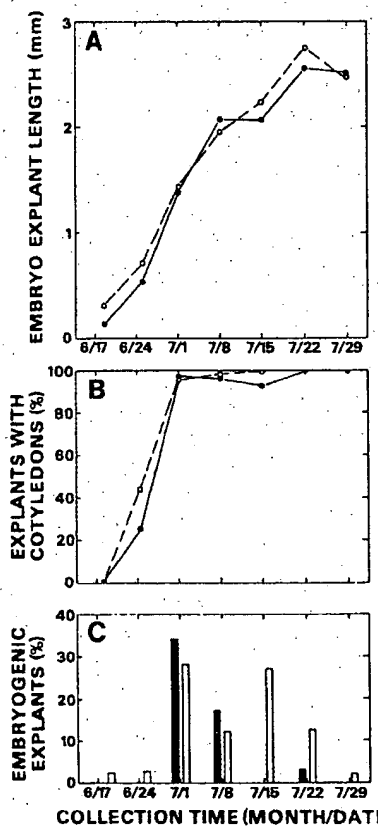

FIGURE 1. The stage of development of white spruce immature embryo explants: (A & B) and the frequency of initiation of EC(C). Solid symbols, tree 1 and open symbols, tree 2.

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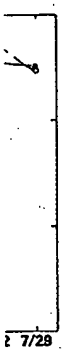

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3.2. Pinus

Two culture protocols were used to initiate EC in Pinus spp. The first protocol used fertilized ovules as an explant, i.e., the female gametophyte with the intact embryo-suspensor complex. This technique was first reported effective for initiation of EC in radiata pine (28). The basal medium used, MSG, was a modified MS basal with NH_4NO_3 replaced by 1450 mg/L (10 mM) glutamine, the KNO_3 level reduced from 1900 to 100 mg/L, and the addition of 745 mg/L of KCl. The basal medium was supplemented with 1% activated charcoal. The second protocol used was the culture of isolated immature embryos. The basal media used were MSG and DCR1. DCR1 was a modified DCR medium used by Gupta and Durzan (11) with the glutamine level increased from 50 to 250 mg/L. Various levels of 2,4-D and BA were added to the basal media on which immature embryos were cultured. All cultures were grown in the dark at 23°C.

3.2.1. Pond pine. Figure 2A shows extruded callus from a loblolly pine ovule. Pond pine extruded callus was phenotypically similar. The frequency at which a white to translucent and mucilaginous callus was extruded from the archegonial end of ovules of pond pine is shown in Table 2. Although up to 12% of the ovules initiated the extruded callus phenotype, only some of these cultures could be maintained (Table 2). Initiation, as used here, refers to initial formation of the extruded callus, whereas maintenance refers to lines established in culture for over one year. To determine the origin of the extruded callus, ovules were cut open when the callus was removed from the ovule for subculture. In all cases the primary embryo had neither developed or atrophied to a significant degree, and callus proliferation was confined to the suspensor region. Histological examination of the extruded callus revealed a mixture of unaggregated suspensor-like cells and globular clumps of densely cytoplasmic cell which resembled pre-embryonal masses (Figure 2B). The majority of the globular structures appeared similar to somatic embryos lacking suspensors. Further histological examination of the extruded callus revealed the presence of early stage somatic embryos, containing an embryonal mass and attached suspensor-like cells (Figure 2C).

TABLE 2. Initiation and maintenance of mucilaginous callus extruded from ovules of pond pine.

Collection time (month/date)	Germination[1] (%)	Extruded callus	
		Initiation[2] frequency (%)	Lines maintained
7/11	0	12	0
7/18	8	2	2
7/25	68	5	1
8/1	96	2	1
8/8	--	1	0

[1]Germination efficiency of embryos cultured on basal medium.
[2]One-hundred ovules cultured per collection.

Smith (28) reported that the frequency at which the extruded callus was formed was dependent on the stage of embryo development. Specifically, his

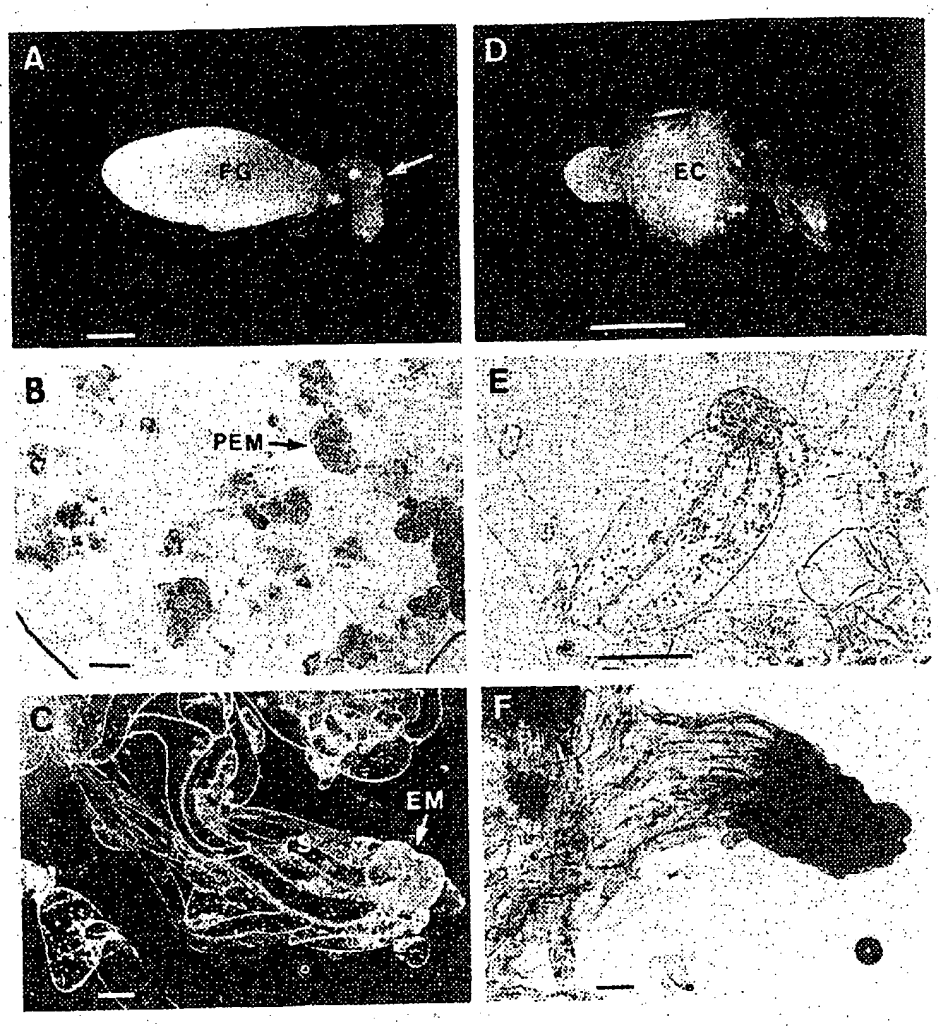
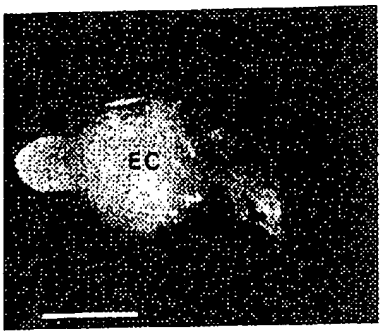
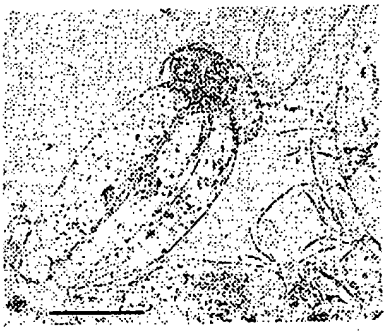
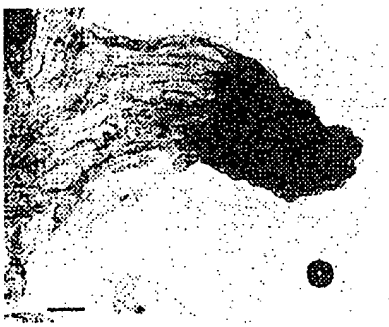


FIGURE 2. Initiation of somatic embryogenesis in Pinus. A: White-mucilaginous callus extruded (arrow) from female gametophyte (FG) of loblolly pine. B: Micrograph of extruded callus of pond pine showing pre-embryonal masses (PEM). C: Early stage somatic embryos of pond pine showing embryonal mass (EM) and attached suspensor-like cell(S). D: Origin of embryogenic callus (EC) from suspensor region of loblolly pine embryo. E: Early stage somatic embryo of loblolly pine. F: White pine somatic embryo. Scale bars: 1 mm in A and D; 100 μm in B, C, E, and F.







sis in Pinus. A: White-mucil-
gametophyte (FG) of loblolly
pond pine showing pre-embryonal
os of pond pine showing
like cell(S). D: Origin of
on of loblolly pine embryo. E:
. F: White pine somatic
m in B, C, E, and F.

results showed that as embryos matured within the ovule (and embryo germination was possible) the initiation frequency dropped off. In pond pine the extruded callus was initiated from both ovules of early collections which contained predominantly pregerminable embryos and ovules of collections as late as August 1, which contained germinable embryos (Table 2).

3.2.2. Loblolly pine. The time course of embyro development in loblolly pine during the period in which cultures were initiated is shown in Figure 3. Note that embryo explants cultured on July 14 to 28 were predominantly precotyledonary and had a mean length of 1 mm or less, whereas embryos cultured during August were mostly cotyledonary and 1 mm or larger.

The stage of explant development was one of the most critical factors for successful initiation of EC from immature embryos of Pinus. The results of our initial experiments (Table 3) showed that EC was only established from precotyledonary embryos which were less than 0.3 mm in length. Subsequent experiments have verified that the precotyledonary immature embryo stage is optimum for initiation and establishment of EC. Initial results also showed that low levels of 2,4-D were effective for initiation of EC, whereas higher levels (10 mg/L) were ineffective (Table 3). The EC originated from the suspensor region of the loblolly pine embryos (Figure 2D), in agreement with the site of origin of EC reported for sugar pine (13). Histologically, the EC callus initiated from immature embryos of loblolly pine was similar to the extruded EC callus of pond pine, i.e., a heterogeneous mixture of unaggregated suspensor-like cells, globular clumps of highly cytoplasmic cells, and early stage somatic embryos (Figure 2E).

3.2.3. White pine. The time course of embryo development during culture initiation in white pine is shown in Figure 4. Note that embryo explants derived from the first two collections of cones (July 2 and 9) were mostly precotyledonary and less than 1 mm in length, whereas those from the last three collections were mostly cotyledonary and greater than 2 mm.

The results in Table 4 show that precotyledonary embryos of white pine (e.g., embryos from the first two collections) were effective explants for initiation of EC, whereas cotyledonary embryos were ineffective. The EC originated from the suspensor region of the immature embryo explants. Thus, initiation of EC in white pine was similar to loblolly with respect to optimal stage of explant development and site of origin. Although EC was initiated on both MSG and DCR basal medium supplemented with 2,4-D and BA (Table 4), no EC was initiated on the DCR 3/0 treatment (data not shown), suggesting a cytokinin requirement for initiation. A white pine somatic embryo is shown in Figure 2F.

3.3. Comparison of *in vitro* embryogenesis in Pinus and Picea

A visual inspection of EC in Pinus and Picea suggests phenotypic similarity. They are both white to translucent and mucilaginous. Further comparative observations of Pinus and Picea EC show marked differences in initiation and growth characteristics.

The most striking differences in initiation include differences in the optimum stage of explant development, the site of origin, and the frequency of initiation of EC. In Picea postcotyledonary embryos were optimum and the EC originated from the hypocotyl (25) and cotyledon regions (22,23). In Pinus precotyledonary embryos were optimum and EC originated from the suspensor region. Initiation frequencies were relatively high in Picea, 40 to 75% from immature embryos and 25% from mature embyros. In contrast, initiation frequencies were consistently low in Pinus, typically less than 3%. It is possible that changes in medium components may increase initiation frequencies in Pinus and enable initiation from more mature

tissues. As indicated previously, media modifications were effective in extending the window to mature embryos in Norway spruce. Another difference between Pinus and Picea was that auxin and cytokinin were obligatory for initiation in Picea but were not required for initiation of EC from fertilized ovules of pond pine.

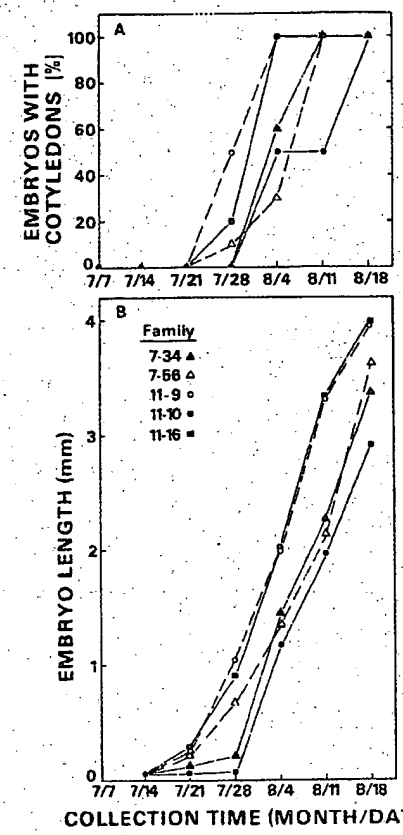


FIGURE 3. The stage of development of immature embryo explants of five families of loblolly pine. A: Percentage of embryos with cotyledonary primordia. B: Embryo length.

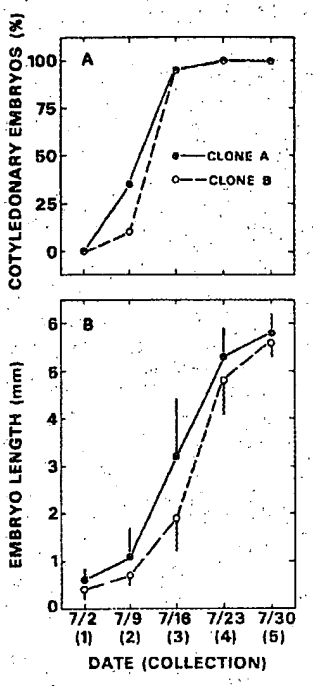


FIGURE 4. The stage of development of immature embryo explants of white pine collected during July 1986 in Wooster, OH. A: Percentage of embryos with cotyledonary primordia. B: Embryo length.

Proliferative growth of EC also differs considerably between Pinus and Picea. In Pinus spp., EC was often dominated by pre-embryonal masses (see Figure 2B) or very early stage somatic embryos (see Figure 2C and E). However, in Picea spp. somatic embryos reached a more advanced stage of development on proliferation medium.

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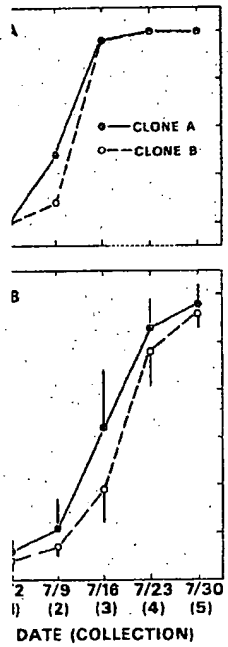

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TABLE 3. Effect of the stage of explant development and media modifications on establishment of EC from immature embryo explants of loblolly pine.

Stage of explant development[1]			Frequency (%) of embyrogenic lines maintained			
			Basal medium (A/C mg/L)[3]			
embryo size (mm)	embryos with cotyledons (%)	N[2]	DCR 10/.5	DCR 3/.5	MSG 10/1	MSG 2/1
0.1-0.3	0	130	0	2.3	0	1.5
0.7-1.2	37	85	0	0	0	0

[1]Embryo explants derived from cones collected July 21 to Aug. 4, 1986 from five families of loblolly pine. See Figure 3.
[2]N = number of explants cultured per each medium treatment.
[3]Refer to text for description of basal medium. A/C refers to auxin and cytokinin levels.

TABLE 4. Effect of the stage of embryo development on initiation of EC in white pine.

Collection (month/date)	Embryos with Cotyledons (%)	N[2]	Embryogenic callus[1] initiation frequency (% explants)	
			Medium	
			MSG 2/1	DCR 3/.5
1 (7/2)	0	150	--	2.7
2 (7/9)	23	230	1.3	2.2
3 (7/16)	95	150	0	0

[1]Initiation from cones stored at 4°C for 2-3 months. Cones were derived from five trees: clones A and B (Figure 4) collected in Wooster, OH and three from Freedom, WI.
[2]N = number of explants cultured per each medium treatment.

4. BIOCHEMICAL CHARACTERIZATION OF EMBRYOGENIC CONIFER CALLUS

Biochemical analyses of Picea and Pinus EC and NEC were conducted as previously described (33-35). Briefly, ethylene was determined by gas chromatography, protein synthesis was measured by 3H-leucine uptake, glutathione (GSH) was measured by the rereduction of oxidized GSH by commercial GSH reductase, and the level of total reductants was measured by the ability to reduce potassium ferricyanide. In Picea spp., EC and NEC assayed were derived from the same genotype (explant) and were cultured under identical conditions. In Pinus spp., although EC and NEC were initiated and maintained under similar or identical conditions, the callus types were derived from different embryo explants, and were therefore genotypically different.

Significant differences in biochemical parameters were detected between EC and NEC in both Pinus and Picea spp. (Table 5). These differences were of sufficient magnitude to suggest that they are indicative of an embryo-

genic condition in conifers. Although interspecific differences were detected, relative to NEC, EC 1) evolved less ethylene, 2) contained lower amounts of GSH and other nonspecific reducing agents, and 3) synthesized or "turned over" protein at a faster rate. Similar trends in protein synthesis rates and level of total reductants between EC and NEC of European larch have also been observed (data not shown). These results suggest that EC callus in conifers is biochemically and metabolically similar irrespective of species, and distinctly different from NEC. While these conclusions are broadly stated for conifers, white spruce stood out as not exhibiting clear biochemical differences between EC and NEC. This may in part be due to the fact that when grown under proliferative conditions, somatic embryos of white spruce can contain anthocyanins which would reduce potassium ferricyanide.

TABLE 5. Biochemical differences between EC and NEC of conifers.

Species	Ratio of measured parameter (NEC/EC)[1]			
	Protein synthesis rate	Total reductants	GSH	Ethylene evolution rate
loblolly pine	0.03	4	9	345
white pine	0.02	13	3	127
pond pine	0.4	10	8	14
Norway spruce	0.03	17	3	12
white spruce	0.4	2	1.2	2.4

[1]Only white spruce ratios not significantly different ($p = 0.05$) from 1.0.

The biochemical assays performed were all rapid, convenient, and required small amounts of tissue. In the case of ethylene, the assay was nondestructive, enabling reuse of the tissue in other experiments. These attributes make the assays attractive candidates for markers of embryogenic potential. However, biochemical markers may have limited utility in conifers because the EC phenotype can be easily recognized. It seems that biochemical assays of EC will be of greater utility in identifying key metabolic pathways involved in growth and development of somatic embryos into plants.

5. ULTRASTRUCTURAL CHARACTERIZATION OF EMBRYOGENIC CONIFER CALLUS

The chloroplasts of light grown conifers are typical of those found in most higher plants. The organelles, usually 2-10 μm in length, contain starch grains, protein bodies and internal thylakoid membranes organized into grana. The organization of the thylakoid membranes is dependent, however, upon the age and physiological state of the tissue.

In contrast, plastids in EC of spruce had a unique morphology at the ultrastructural level (Figure 5A & B). The plastids, appeared more darkly stained (more electron dense) than mitochondria, lacked the internal organization of a mature chloroplast. Some of these plastids contained small starch grains, although they were not nearly as large as the starch grains present in mature chloroplasts in leaf or cotyledon tissue. The plastids present in the green NEC of spruce, on the other hand, appeared similar to a typical chloroplast (Figure 5C & D). Thylakoid membranes, some organized into grana, were found in all the chloroplasts. Large starch grains were also prominent in some of the chloroplasts.

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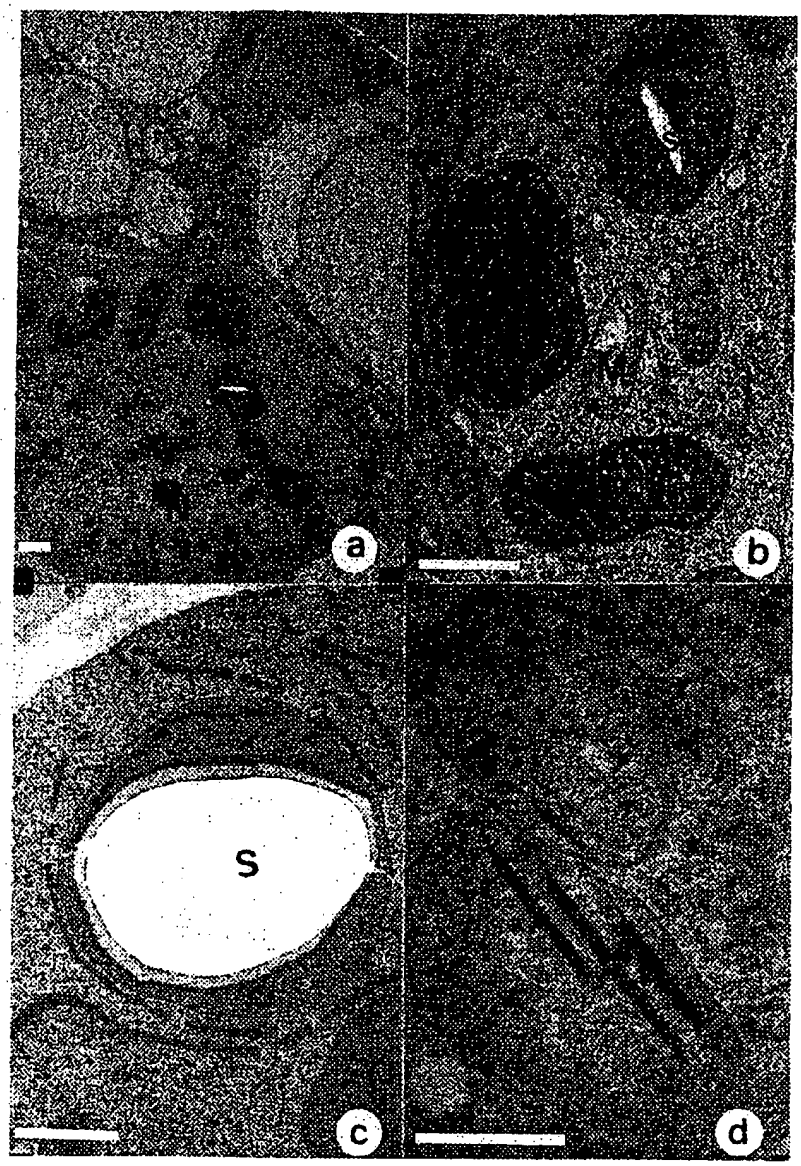

FIGURE 5. Proplastids in embryogenic (A & B) and chloroplasts in non-embryogenic (C & D) callus of Norway spruce. P = proplastid. M = mitochondria. S = starch grain. G = grana. N = nucleus. Scale bars = 1 μm.

Zygotic embryos excised from mature Norway spruce seeds were also examined. The plastids resembled those observed in EC of spruce. That is, they also lacked organized thylakoid membranes (photographs not shown). In order to determine if the plastid morphology observed in zygotic and somatic embryos was unique to Norway spruce, EC of loblolly pine, pond pine, white pine, and European larch were examined (10). EC of these species all contained plastids that closely resembled those in EC of spruce (photographs not shown). In addition, the plastids observed in somatic embryos of carrot exhibited the same morphology as those in EC of conifers.

Further investigations showed that as Norway spruce somatic embryos matured (cotyledons begin to form and green) chloroplasts with thylakoids and grana became evident. We have observed the same phenomenon in carrot, where somatic embryos beyond the torpedo stage, if grown in the light, contained green mature chloroplasts. Thus, it appears that proplastids are indicative of early stages of embryonic development, whether *in vivo* or *in vitro*. These findings provide evidence in support of our previous biochemical studies (9,33,34) that many aspects of somatic embryogenesis mimic or correspond to *in vivo* embryogenesis (21). In summary, chloroplast morphology may be one of the best indicators that embryogenesis in *Pinus* and *Picea* is occurring and proceeding "normally" *in vitro*.

6. CONIFER EMBRYOGENIC SUSPENSION CULTURES

For the purposes of mass propagation, a liquid embryogenic culture system is desirable for several reasons, including the following. Liquid cultures are easier and more economical to maintain than callus cultures. They have potential for higher growth rates (decreased cell doubling times), thus eliminating the need for maintaining large stocks of callus. Lastly, liquid culture systems are amenable to automation of both somatic embryo proliferation and development *en masse* (e.g., large bioreactor culture systems). In addition to their utility for mass propagation, liquid cultures serve as an ideal source for cells and protoplasts for genetic modification experiments.

Although establishment of embryogenic suspension cultures has been reported in both *Picea* and *Pinus* (14,18), quantitative information has not been presented on growth characteristics and somatic embryo yield. We have established rapidly growing embryogenic suspensions of Norway spruce. The cultures were derived from EC initiated from mature embryo explants on half-strength BLG with 2 mg/L 2,4-D or NAA and 1 mg/L BA as previously described.

During the linear phase of growth, the liquid cultures had a doubling time of about 48 hours and reached a somatic embryo density of about 100/mL (Figure 6). Rapid growth rates were maintained by repeated subculture at 10-14 day intervals (1:10 dilution with fresh medium).

With both high growth rates and somatic embryo yield, the Norway spruce suspension cultures show promise as an ideal system for mass production of somatic embryos. Continued efforts are directed at obtaining maturation of the somatic embryos while still in the liquid culture system.

7. DEVELOPMENT OF CONIFER SOMATIC EMBRYOS TO PLANTS

The Norway spruce somatic embryos can be converted to phenotypically normal plants (Figure 7). Somatic and seedling plants overwintered and renewed vegetative growth synchronously. A complete description of the frequency of plant regeneration is presented elsewhere (6). Based on optimum maturation frequencies (25%), germination (56%), and conversion to plants (29%), the highest mean efficiency of 4% was attained. Because of

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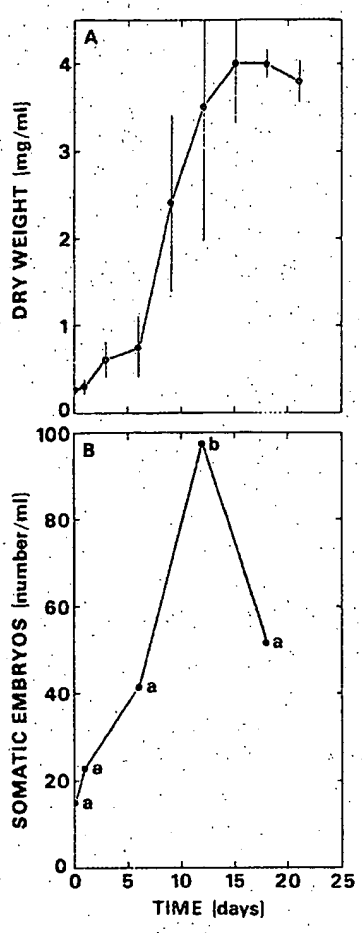

FIGURE 6. Growth curve (A) and somatic embryo density (B) of liquid suspension cultures of Norway spruce grown on half-strength BLG with 2 mg/L NAA and 1 mg/L BA. Vertical bars in A are ± standard deviation among triplicate samples. Means of triplicate counts in B followed by unlike letters are significantly different ($p = 0.05$).

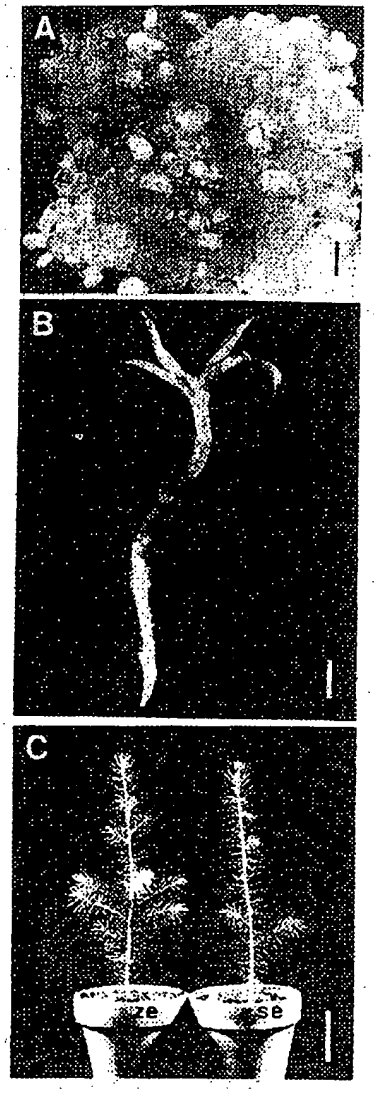

FIGURE 7. Maturation (A), "germination" (B), and conversion (C) of Norway spruce somatic embryos to plants. C: Comparison of somatic embryo plant (se) and control plant grown from zygotic embryo (ze). Scale bars: 1 mm in A & B; 5 cm in C.

the exceedingly long regeneration cycles of conifers, it is of prime importance to verify at an early stage that the somatic plants are uniform and genetically "true-to-type." Current efforts are directed toward production of large numbers of Norway spruce somatic plantlets for quantitative analysis of uniformity and growth characteristics.

8. COMPARISON OF IN VIVO AND IN VITRO CONIFER EMBRYOGENESIS

Polyembryony, the formation of multiple embryos, is common in vivo in conifers. There are two types of polyembryony, simple and cleavage (8,26) (Figure 8). Simple polyembryony, the fertilization of more than one egg per female gametophyte, occurs in both Picea and Pinus. Therefore, the resulting embryos may be genotypically different. In addition to simple polyembryony, cleavage polyembryony occurs in Pinus (Figure 8). Cleavage embryos result from the division of apical tier cells of an individual proembryo and are, therefore, genetically identical.

Observations of in vitro embryogenesis in both Picea and Pinus have suggested that one mechanism of embryo formation may be a cleavage type of process. That is, multiple embryos were found with common suspensors. Although further investigations are needed to fully characterize possible types of in vitro embryogenesis in conifers, the following suggest the possibility of differences between Picea and Pinus. As indicated previously, in Picea a callus phase preceded initiation of somatic embryos (25). Therefore, in Picea besides cleavage formation of embryos in vitro, another type of embryo initiation mechanism is operative. In Pinus initiation of embryogenesis in vitro may simply be a reinitiation of the cleavage process (which occurs in vivo).

9. SUMMARY

Much progress has been made on the development of in vitro embryogenic systems in conifers since the first reports of somatic embryogenesis in Norway spruce (16) and European larch (24) in 1985. The embryogenic callus phenotype, white to translucent and mucilaginous, has been similar in all conifers. Furthermore, our biochemical studies have provided evidence of the physiological similarity of embryogenic conifer callus among species. Although similar physiologically, EC in Picea and Pinus have different optimum initiation windows and sites of origin from immature embryo explants. These differences may relate to inherent differences in embryology between Picea and Pinus.

Considerable progress has also been made on regeneration of plants from conifer somatic embryos. Several reports have verified that conifer somatic embryos reached the germination stage (3,12-14,17,19,24,30). Our results (6) indicate that Norway spruce somatic embryo plants were similar to seedling derived plants in terms of physiological response to changing environment. This is the first demonstration of overwintering and renewed vegetative growth from resting buds of conifer somatic embryo plants.

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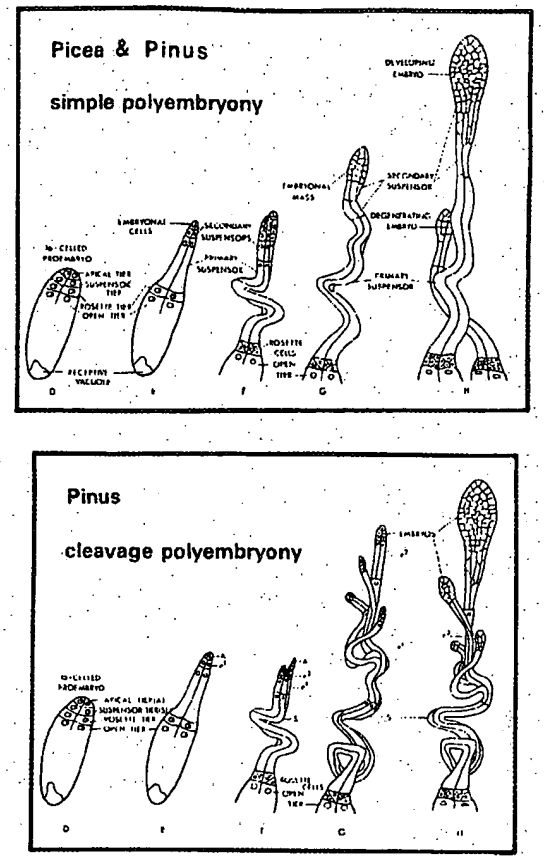

FIGURE 8. In vivo embryogenesis in Picea and Pinus. Used with permission from Owens and Blake (26).

10. ACKNOWLEDGMENTS

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Plant Cell Reports

Initiation of embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus* L.)*

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Abstract. Embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus*) have been obtained. The whole female gametophyte was plated on a medium containing 50 mg/l glutamine, 500 mg/l casein hydrolysate, 3% sucrose, 2 mg/l 2,4-D, 1 mg/l BA and 0.2% Gelrite as a solidifying agent. Embryogenic calli could be seen as early as 5 days following culture. Histological studies indicate proliferation of pre-existing embryogenic tissue in the corrosion cavity followed by extrusion of embryogenic callus through the micropylar end of the gametophyte. Embryogenic suspension cultures were obtained by placing embryogenic callus into liquid medium. Embryogenic suspension cultures were subcultured weekly and proliferated as early-stage embryos with attached suspensors. Embryo development was obtained following transfer of the embryogenic tissue to an auxin-free medium containing 50 mM glutamine, 38 μM abscisic acid, and 6% sucrose. Although embryo development could be consistently obtained, whole plants have not yet been recovered from these somatic embryos.

Abbreviations: 2,4-D = 2,4-Dichlorophenoxyacetic acid, ABA = Abscisic acid, BA = 6-Benzyladenine

Introduction

Over the past few years, much progress has been made in the area of somatic embryogenesis in conifers. Somatic embryogenesis and plant regeneration have been reported for loblolly pine (*Pinus taeda* L.) (Gupta and Durzan, 1987), sugar pine (*Pinus lambertiana* Lamb.) (Gupta and Durzan, 1986), Douglas-fir (*Pseudotsuga menziesii*) (Durzan and Gupta, 1987), Norway spruce (*Picea abies* (L.) Karst) (Hakman and von Arnold, 1985), and both black spruce (*Picea mariana* (Mill.) BSP.) and white spruce (*Picea glauca* (Moench) Voss.) (Hakman and Fowke, 1987a). The explant source in all except one of these reports was the excised, immature or mature zygotic embryo. In one study, Gupta and Durzan (1987) reported use of the whole female gametophyte with attached suspensors and embryos for initiation of embryogenic callus (embryonal-suspensor masses) of loblolly pine. In that study, embryonal suspensor-masses formed in 9 to 10% of the total explants.

The origin of proliferative embryogenic tissue in conifers varies with explant tissue and plant type. Although histological studies on the initiation process have not been performed, the source of embryogenic tissue has been reported to be suspensor tissue in sugar pine (Gupta and Durzan, 1986) and epidermal or subepidermal tissue of the hypocotyl in spruce (Nagmani *et al.*, 1987). Although plant regeneration from somatic embryos of some conifers can be consistently obtained, the efficiency of somatic embryo initiation and development is often very low, especially for pines. This paper describes a system for high frequency initiation of embryogenic callus in eastern white pine (*Pinus strobus*) and the source of that proliferative embryogenic tissue. In addition, development of an embryogenic suspension culture of eastern white pine is described.

Materials and methods

Initiation and Subculture of Embryogenic Callus: Seed cones of eastern white pine (*Pinus strobus* L.) were collected weekly for 6 weeks starting in June of 1988 and continuing to August of 1988. Trees were located near Wooster, Ohio and cones were collected from different eastern white pine trees. The cones were stored in paper bags at 4°C for a maximum of three months prior to explanting. Immature seeds were removed from the cones and surface sterilized for 20 minutes in a 15% commercial bleach solution (0.8% sodium hypochlorite) containing 0.5% Tween-20. After 3 rinses with sterile, distilled water, gametophytes and embryos were excised for culture. The gametophyte refers to the female gametophyte with the intact immature zygotic embryo(s) and suspensor tissue. Small embryos, which were prone to desiccation, were kept moist by performing manipulations in small amounts of liquid medium. Twenty-five explants were placed into each of three 100 x 25 mm Petri dishes containing 35 ml of of DCR-m medium which consisted of DCR basal medium (Gupta and Durzan, 1985) with 50 mg/l glutamine, 500 mg/l casein hydrolysate, 3% sucrose, 2 mg/l 2,4-D, 1 mg/l BA, and 0.2% Gelrite as a solidifying agent. All medium treatments were performed in triplicate and explants were evaluated for production of embryogenic callus weekly for 8 weeks. Cultures were initiated and maintained at 23°C in the dark. Once embryogenic callus cultures were established, cultures were subcultured biweekly.

Histology: Immature gametophytes from 2 collection dates (July 5 and 11, 1988) were placed on DCR-m medium as described above and removed following 0,2,3,4,7,9, and 11 days of culture for histological studies. For each time point and collection date, 25 gametophytes were randomly selected. The gametophytes were fixed in a Formalin-acetic acid-alcohol solution (Berlyn and Miksche, 1976), dehydrated in a tertiary-butyl alcohol/ethanol series and embedded in paraffin according to Johansen (1940). Paraffin sections were cut 13 to 40 μm thick and stained using Johansen's (1940) quadruple stain.

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Offprint requests to: J. J. Finer

Exhibit 2
Becwar

Embryogenic Suspension Culture: For initiation of embryogenic suspension cultures, 10 to 100 mg of embryogenic tissue was placed into 35 ml of liquid DCR-m in a 125 ml deLong flask. Suspension cultures were agitated at 150 rpm and maintained at 23°C in the dark. Liquid cultures were subcultured every 3 to 4 days to biweekly depending on the quantity of tissue used for subculture. For subculture, 35 ml of fresh medium was added to the flask to be subcultured (total volume now 70 ml). The flask was swirled and 35 ml of the diluted suspension was removed with a pipet and placed in a new flask. If a low inoculum of tissue was used, then subculture was biweekly. Higher inoculum subculture required a 3 to 4 day subculture.

Embryo Development: Embryo development was obtained following transfer of the tissue first to liquid and then solid auxin-free media. For early development in liquid media, cells were first allowed to settle for 5 min in deLong flasks and the old supernatant medium was removed and discarded. The cells were washed 2 times with auxin-free DCR-m medium containing 50 mM glutamine, 38 μM ABA, and 12% sucrose and resuspended in this auxin-free medium. Developing embryos were washed in this liquid development medium weekly for 2 weeks before plating on solid medium.

For further embryo development on solidified media, developing embryos and embryogenic tissues obtained as described were utilized. Clumps of developing embryos (1 to 4 mm) were placed on auxin-free Gelrite-solidified (0.2%) DCR-m media containing all combination of 1, 3, 6, and 12% sucrose; 0, 15, and 50 mM glutamine; and 0, 19 and 38 μM ABA. Twenty-five clumps of embryogenic tissue were placed in each of three Petri dishes at 23°C in the dark. One month after transfer to the solid development medium, the number of developing embryos on each clump of embryogenic tissue was recorded.

Results and Discussion

Initiation of Embryogenic Callus: The gametophyte was clearly the superior explant source for initiation of embryogenic tissue in eastern white pine (Table I). An average of 54% (in one case, 72%) of the gametophytes formed embryogenic callus. For this reason, the gametophyte was used as the explant for all subsequent experiments. In an earlier study on initiation of eastern white pine embryogenic callus, Becwar *et al.* (1988) obtained a maximum of 2.7% of excised eastern white pine embryos that formed embryogenic callus. It was reported that only precotyledonary embryos formed embryogenic callus and the origin of the callus was the suspensor region (Becwar *et al.*, 1988). Although the gametophyte was used as the explant source for initiation of embryogenic tissue in loblolly pine (Gupta and Durzan, 1987), a comparison between gametophytes and excised embryos as explant sources was never made. In that study, 9 to 10% of the gametophytes formed embryogenic callus.

Table I Percent of eastern white pine explants forming embryogenic callus after 8 weeks of culture. Cones for this experiment were collected on July 11, 1988.

Explant	Percent explants forming embryogenic callus ± SE
Excised zygotic embryo	3.0 ± 3.3
Whole gametophyte	54.0 ± 10.8
Corrected gametophyte*	63.8 ± 15.0

*value is percent of gametophytes forming embryogenic callus divided by the percent gametophytes containing at least one nonaborted embryo.

Zygotic embryos in gametophytes from the July 11 collection date ranged from 0.1 to 0.3 mm in length and cotyledon development was apparent in less than 50% of the embryos. Ten to twenty percent of the gametophytes did not contain visible embryos and were considered to be aborted. Since some of the gametophytes did not contain embryos, a corrected value for the gametophyte based on the presence of at least one nonaborted embryo is presented in Table I. This corrected gametophyte value may be used to better compare the values obtained for gametophyte versus excised embryos. The maximum frequency of 72% reported earlier translates to a corrected gametophyte value of 89%.

Embryos which were excised for culture from cones from later collection dates (July 18, July 25, August 1, 1988) produced embryogenic callus at a frequency of less than 1%. Embryos from the July 11, 1988 collection date were less than 0.3 mm in length and therefore very difficult to excise. The advantages of using the whole gametophyte for establishment of embryogenic cultures of eastern white pine are two-fold. First, the frequency of embryogenic callus initiation is much higher than that obtained with the excised embryo (Table 1) and second, it is simply much easier to culture the gametophyte and not attempt dissection of a very small embryo.

Embryogenic callus was observed protruding from the micropylar end of the gametophyte as early as 5 days following culture. The embryogenic callus was mucilagenous and translucent (Fig. 1) as is typical of embryogenic tissue of other conifers (Hakman and von Arnold, 1985). Stage 2 somatic embryos (Hakman and Fowke, 1988) were only rarely observed on the embryogenic callus. Numerous embryo initials, which consisted of 4 to 20 cells and were attached to elongated suspensor cells, were observed protruding from the callus. Approximately two-thirds of the embryogenic calli proliferated following removal from the gametophyte. The other one-third may represent suspensors and zygotic embryos which were simply extruded through the gametophyte.

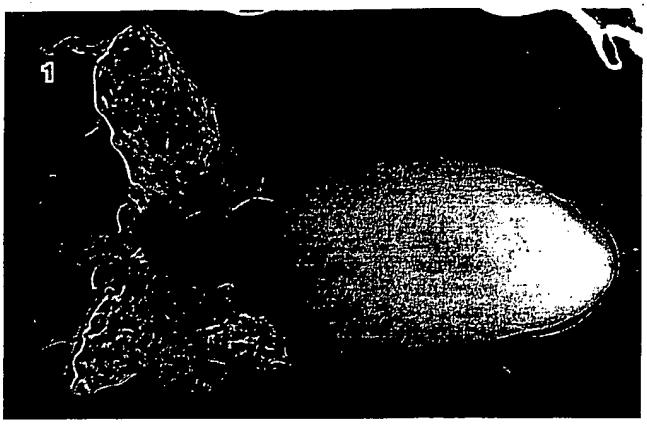
The influence of embryo age or state of maturation on initiation of embryogenic callus is shown in Table II. Gametophytes which were taken from cones that were harvested on the earliest collection date gave the lowest percent of embryogenic callus. We estimate on the basis of previous research (Kriebel, 1972) that fertilization date for these cones was between June 16 and June 24, 1988. The highest percentage of initiation of embryogenic callus was obtained from the cones with the more mature (but still young) gametophytes that contained mostly precotyledonary zygotic embryos.

Table II Effect of embryo age on initiation of embryogenic callus from whole gametophytes of eastern white pine.

Cone Collection Date*	Percentage gametophytes forming embryogenic callus
June 20, 1988	6.0 ± 2.0 (± SE)
June 27, 1988	32.0 ± 5.7
July 5, 1988	48.0 ± 2.8
July 11, 1988	54.0 ± 10.8

*Estimated fertilization date = June 20 ± 4 days.

Histology: Determination of the origin of somatic embryogenic tissue in eastern white pine gametophytes was difficult due to the large number of zygotic embryos present in the gametophyte before culture. In one case, 8 small zygotic embryos were observed in a single fertilized female gametophyte (Figs. 2 and 3 show 2 different sections of the same gametophyte containing 8 small zygotic embryos). It is not unusual in pine to have a large number of zygotic embryos although usually only one develops



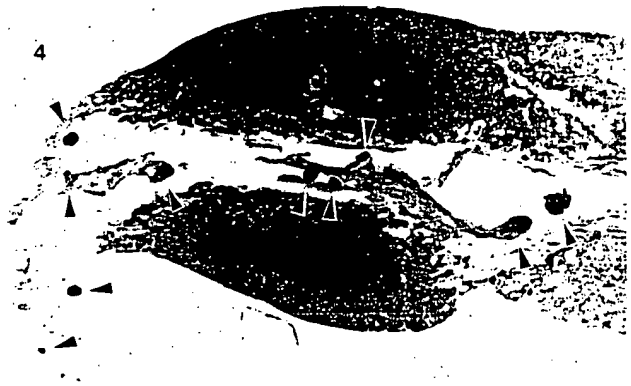


Fig. 1 Production of embryogenic callus through micropylar end of female gametophyte (X 7.2). Fig. 2 Section through female gametophyte before culture showing 3 zygotic embryos (X 37). Fig. 3 Different section through gametophyte shown in Fig. 2 showing presence of 5 additional zygotic embryos (arrows) (X 38). Fig. 4 Section of gametophyte placed in culture for 9 days showing extrusion of embryogenic callus from the micropylar end of the female gametophyte. Ten embryos (arrows) can be seen in this section (X 19).

to maturity (Buchholz, 1918). Cleavage polyembryony can result in as many as 48 zygotic embryos in fertilized immature seed of pine (Buchholz, 1918).

The staining properties of zygotic and somatic embryos were similar using the quadruple staining procedure of Johansen (1940). It was not possible to distinguish between the small zygotic embryos and proliferating somatic embryos in the gametophyte based on this histological study. Figure 4 shows a section of a gametophyte which had been placed in culture for 9 days. Embryogenic callus can be seen protruding from the micropylar end of this gametophyte and 10 embryos can be observed in this single section. The first somatic embryos in eastern white pine may simply result from proliferation of smaller zygotic embryos, which would otherwise abort during development under natural conditions. The low percentage initiation of embryogenic callus resulting from use of single excised zygotic embryos (Table I) may be a result of discarding the smaller zygotic embryos. It was not possible to separately culture the smaller abortive embryos and the large surviving zygotic embryo.

Embryogenic Suspension Cultures: Embryogenic suspension cultures of eastern white pine were not difficult to initiate and maintain in DCR-m medium. Due to the presence of long suspensor cells (Fig. 5) on the embryogenic tissue, tissue in suspension culture appeared almost fibrous in nature. The embryo proper consisted of a small cluster of densely cytoplasmic cells that appeared yellow-brown when viewed on an inverted microscope. The suspensors were highly elongated and vacuolated. As reported in other studies (Hakman and Fowke, 1987b), the suspensors were attached to small embryos and were observed to occur as single strands or in groups of parallel strands.

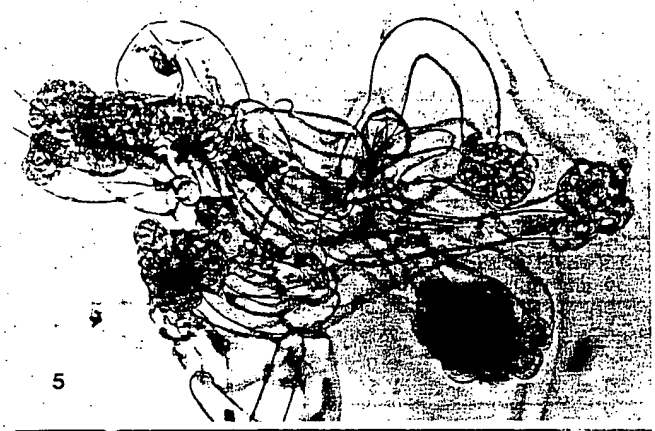


Fig. 5 Embryogenic suspension culture tissue of eastern white pine (X 66). Fig. 6 Stage 2 somatic embryo on solid medium (X 34).

Liquid cultures have been maintained for 6 months using low density subculture. As little as one clump (~2 mg fresh weight) of embryogenic tissue could be used for subculture. Low inoculum subculture has been used successfully for maintenance of embryogenic suspension cultures of cotton (Finer, 1988), soybean (Finer and Nagasawa, 1988), and Chinese yam (Nagasawa and Finer, 1989). If subculture was delayed or the density of the cells became greater than 0.5 g fresh weight/35 ml medium, the quality of the embryogenic suspension culture rapidly declined. The necessity of frequent subculture of embryogenic tissue of pine has previously been reported (Gupta and Durzan, 1987).

Somatic Embryo Development: Following transfer of embryogenic suspension culture tissue to a liquid auxin-free DCR-m medium containing 50 mM glutamine, 38 μM ABA, and 12% sucrose, the embryos underwent early development. Clumps of stage 1 embryos (Hakman and von Arnold, 1988), that were easy to transfer with a forceps, were formed in 2 weeks. These clumps of early-staged developing embryos were used as a uniform inoculum for development studies.

In all cases, clumps of embryogenic tissue that were plated on solidified media lacking glutamine proliferated as embryogenic callus. This embryogenic callus contained no stage 2 embryos. Stage 2 embryos (Fig. 6) were formed following the addition of 50 mM glutamine, 6% sucrose, and 38 μM ABA (Table III). These embryos were light-yellow to cream colored and were supported by suspensor tissue.

Table III Number of eastern white pine somatic embryos formed per plate after 30 days. (± SE)

Sucrose and glutamine concentrations	ABA concentration (μM) 0	19	38
1% Sucrose			
0 mM glutamine	0	0	0
15 mM glutamine	0.7 ± 0.9	2.7 ± 1.2	0.3 ± 0.3
50 mM glutamine	1.0 ± 0.8	4.3 ± 4.0	2.3 ± 2.6
3% Sucrose			
0 mM glutamine	0	0	0
15 mM glutamine	1.3 ± 1.2	0.3 ± 0.5	0.3 ± 0.3
50 mM glutamine	1.3 ± 1.2	5.7 ± 3.1	10.3 ± 7.4
6% Sucrose			
0 mM glutamine	0	0	0
15 mM glutamine	1.0 ± 1.4	0.7 ± 0.9	1.7 ± 2.4
50 mM glutamine	4.7 ± 1.7	10.3 ± 3.8	23.3 ± 4.2
12% Sucrose			
0 mM glutamine	0	0	0
15 mM glutamine	1.0 ± 1.4	4.0 ± 2.2	6.3 ± 8.3
50 mM glutamine	1.5 ± 0.5	4.3 ± 4.0	13.3 ± 5.4

In treatments where embryo development was not observed, proliferation of embryogenic callus was usually apparent. Media containing 12% sucrose and 0 or 19 μM ABA at all glutamine levels were the exceptions. On these media, the embryogenic tissue turned bright yellow but growth was minimal. Growth and development were also retarded on media containing 150 mM glutamine (data not shown). The best medium for embryo development to stage 2 contained 50 mM glutamine, 6% sucrose, and 38 μM ABA.

Although the effects of glutamine on conifer somatic embryo development have not been previously quantified, glutamine, at lower concentrations, has been used successfully for development of sugar pine (~1.4 mM) (Gupta and Durzan, 1986) and loblolly pine (~3.1 mM) (Gupta and Durzan, 1987) somatic embryos. Higher levels of glutamine have also been reported to be beneficial for somatic embryo development in alfalfa (30 mM) (Stewart and Strickland, 1984) and cotton (15 mM) (Finer, 1988).

Abscisic acid at 38 μM promoted embryo development to stage 2 in eastern white pine. Enhancement of conifer somatic embryo development by ABA addition has previously been reported for white spruce (Hakman and von Arnold, 1988), Norway spruce (Becwar *et al.*, 1987; Boulay *et al.*, 1988; von Arnold and Hakman, 1988) and Douglas-fir (Durzan and Gupta, 1987). In eastern white pine, this promotion was enhanced by raising the sucrose concentration from 3% to 6% (Table III). Hakman and von Arnold (1988) also reported interactions between ABA and sucrose in white spruce embryo development and subsequent germination from embryogenic suspension culture material. Lu and Thorpe (1987) showed by sorbitol substitution that the sucrose enhancement of embryo development in their white spruce system was an osmotic effect. They reported no improvement of somatic embryo development by addition of ABA.

This paper reports on the efficient production of embryogenic callus and suspension cultures of eastern white pine (*P. strobus*). Embryos obtained using the procedure described in this paper have recently developed to stage 3 following transfer to a growth regulator-free medium. Efforts are underway to induce these embryos to germinate and form whole plants.

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OPTIMIZED SOMATIC EMBRYOGENESIS IN *PINUS STROBUS* L.

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Summary

Somatic embryogenesis (SE) initiation in *Pinus strobus* was optimized by the manipulation of plant growth regulator (PGR) concentrations in the culture medium. Modified Litvay medium (MLV) of Litvay et al. (1985) supplemented with lower than routinely used PGR concentration increased initiation of established embryogenic cultures from approximately 20 to 53%. The original developmental stage of zygotic embryos had a pronounced effect on the SE response. The optimum stage was the pre- to shortly post-cleavage stage. A substantial genetic influence on initiation of SE was indicated by a significant variance component due to families. Genotype × collection date and genotype × media interactions had large effects on initiation of SE. The PGR levels in the culture medium prior to maturation had a significant effect on subsequent production of mature somatic embryos. Embryogenic tissue initiated and proliferated on medium with a low level of PGR consistently produced a high number of somatic embryos, indicating that optimized initiation protocol also enhanced somatic embryo production. Somatic embryos of 93 embryogenic lines (representing five families) that were initiated on media with different PGR concentrations were converted to plants at an overall frequency of 76%, and grown in the greenhouse. With these improved protocols, application of *P. strobus* SE in commercial clonal forestry is feasible as an alternative to traditional breeding and reforestation.

Key words: conversion to plants; eastern white pine; embryogenic cultures; plant growth regulators.

Introduction

Somatic embryogenesis (SE) in *Pinus strobus* L. (eastern white pine) has been reported by Becwar et al. (1988), followed by Finer et al. (1989) and Kaul (1995). In these reports, initiation of established embryogenic cultures from immature zygotic embryos ranged from 3 to 36%. More recently, Klimaszewska and Smith (1997) and Garin et al. (1998) obtained established somatic embryogenic lines at frequencies of 15 and 2%, respectively. The latter was an average obtained with 13 open-pollinated (OP) families. These SE initiation rates, although sufficient to generate genetically different embryogenic cultures for experimentation, are too low for commercial application in clonal propagation of forest tree species, as high numbers of genotypes from a given seed family are necessary for selection, maintenance of genetic diversity, and clonal field tests. In spruce species such as *Picea glauca* and *P. abies*, SE initiation frequency is above 50% from immature embryos (Park et al., 1993; Högberg et al., 1998). It is apparent that improvements in the protocol for increased initiation are necessary for commercial application of SE in *Pinus strobus*.

The results published to date, for pines (Handley et al., 1995; Garin et al., 1998; Lelu et al., 1999) and other conifers (Cheliak and Klimaszewska, 1991; Park et al., 1993), showed that SE initiation was under a strong genetic influence. In *Picea glauca*, such a genetic influence was primarily due to additive genetic effects, accounting for an average of 42% of the total variance. The SE initiation frequency was, on average, 56% from immature embryos and 26% from mature embryos among 30 control-pollinated (CP) families used (Park et al., 1993). To determine if a similar genetic effect existed in *Pinus strobus*, a SE initiation protocol that would provide an initiation response high enough to allow for appropriate statistical analysis was needed.

In most pines, SE initiation is limited to the first few weeks of embryo development prior to the emergence of cotyledonary primordia (Finer et al., 1989; Becwar et al., 1990; Lelu et al., 1999; Park et al., 1999; Salajova et al., 1999; Percy et al., 2000). Hence targeting the most responsive stage of embryo development is an important parameter for SE initiation in any pine species.

Another factor pertinent to SE in conifers is the composition of the initiation medium. Various media have been used to culture immature zygotic embryos of pines, the most common of which are DCR (Gupta and Durzan, 1985), Litvay (Litvay et al., 1985; Nagmani and Bonga, 1985) and MSG (Becwar et al., 1990). Typically, these media were supplemented with both L-glutamine and casein hydrolysate or with L-glutamine alone as the only source of organic nitrogen. The most commonly added plant growth regulator (PGR) combination in conifer SE, particularly in spruce and pine species, has been 2,4-dichlorophenoxyacetic acid (2,4-D

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Exhibit 3
Becwar

at 9.5–15 μ*M*) and 6-benzyladenine (BA at 2.25–5.0 μ*M*). In the results published to date on *P. strobus* SE, all the above-mentioned media have been tested with a single concentration of PGR, i.e., 9.5 μ*M* 2,4-D and 4.5 μ*M* BA (Finer et al., 1989; Kaul, 1995; Klimaszewska and Smith, 1997; Garin et al., 1998).

One approach to improving SE yields may be to manipulate the PGR concentration in the initiation medium in conjunction with the use of various genotypes at different stages of embryo development.

This study had three main objectives. The first was to determine the optimal concentration of PGR to increase the initiation rate of SE in five OP families of *P. strobus* collected at five dates during the development of zygotic embryos on one nutrient medium. The second objective was to test the effect of optimized PGR concentration on the SE initiation rate of a wider range of OP families collected at the same and extended dates on two nutrient media. The third objective was to determine the effect of PGR levels in initiation and proliferation media on subsequent somatic embryo maturation capacity of embryogenic lines. The overall conversion rate of somatic embryos to plants was determined.

Materials and Methods

Plant material. Cones of *P. strobus* L. (eastern white pine) were collected from the Nova Scotia Tree Improvement Working Group's (NSTIWG) clonal seed orchard in Debert, Nova Scotia, Canada, during the summer of 1999. A total of 14 seed trees were selected depending on the availability of cones. Open-pollinated cones from each of the 14 trees (OP families) were collected weekly for six collection dates from June 28 to August 2. At each collection date, samples of randomly selected seeds (15–20) from each of the five OP families (NSTIWG clone numbers 1053, 1054, 1055, 1073, and 1148) were stained with 1% potassium iodide (IKI) (Jensen, 1962) and examined microscopically to determine the developmental stage of zygotic embryos (Table 1).

For culture initiation, seeds extracted from cones were placed in metal baskets and stirred vigorously for 6 min in 200 ml of 3% (v/v) hydrogen peroxide with one drop of Tween 20. Seeds were then rinsed with sterile water three to four times. Seeds of a given family were then transferred to a Petri dish containing a moist, sterile filter paper. The seed coat, nucellus, and membrane surrounding the megagametophyte were removed under the stereomicroscope, and the megagametophyte was placed on the initiation medium in a 100 × 15 mm Petri dish.

TABLE 1

DESCRIPTION OF MEGAGAMETOPHYTES AND DEVELOPMENTAL STAGES OF ZYGOTIC EMBRYOS OF *PINUS STROBUS* AT EACH COLLECTION DATE (IN 1999)

Collection number and date	Developmental stage[a]
1. June 28	Megagametophytes translucent. Embryos at the pre-cleavage stage.
2. July 5	Megagametophytes slightly opaque. Embryos mostly at the pre-cleavage stage. One family slightly more advanced.
3. July 12	Megagametophytes slightly opaque to opaque. Embryos at the post-cleavage stage with little or no dominance; i.e., one embryo distinctly larger than others. A few multiple fertilizations observed.
4. July 19	Embryo dominance established.
5. July 26	Single, dominant embryo at the pre-cotyledonary stage.
6. August 2	Embryos with cotyledons.

[a] The embryos were dissected from the megagametophyte under a stereomicroscope in a drop of IKI solution. The stain accumulated in the embryo cells, thus permitting determination of its developmental stage.

Experiment 1: Optimization of PGR concentration for SE initiation. This experiment was conducted at the Canadian Forest Service (CFS), Laurentian Forestry Centre, Sainte-Foy, Quebec, Canada. The plant material included five of the 14 OP families from collections 2–6 (from July 5 to August 2).

The nutrient medium consisted of a modified Litvay's (Litvay et al., 1985) medium (MLV) with half-strength macro-elements and full-strength micro-elements and Fe-EDTA. The medium was supplemented with 1 g l^{-1} casein hydrolysate (Difco, Detroit, MI, USA), 0.5 g l^{-1} L-glutamine, 20 g l^{-1} sucrose (Sigma Chemical Co., St. Louis, MO, USA), and was solidified with 4 g l^{-1} gellan gum (Phytagel, Sigma). A solution of L-glutamine was adjusted to pH 5.8 and filter-sterilized aliquots were added to the autoclaved molten medium. The pH of the medium was adjusted to 5.8 prior to sterilization. The following three PGR concentrations were evaluated: 'S-PGR', a standard PGR medium containing 9.5 μ*M* 2,4-D and 4.5 μ*M* BA; 'L-PGR', a low PGR medium containing 2.2 μ*M* each of 2,4-D and BA; and 'UL-PGR', an ultra-low PGR medium containing 1.1 μ*M* each of 2,4-D and BA.

Five to eight megagametophytes were cultured in each Petri dish containing approximately 25 ml of medium, and dishes were sealed with Parafilm. Depending on the availability of seeds, 30–85 explants were cultured per family, collection date, and PGR concentration. Thus 5–11 replicates (Petri dishes) per each parameter were used in the subsequent statistical analysis. Cultures were kept in darkness at approximately 24°C for the duration of the experiment.

Frequency of SE initiation was assessed after 12 wk of culture. A presence of distinct, early somatic embryos and their subsequent vigorous growth were the criteria for scoring the outgrowth as an established embryogenic tissue. Data on the number of explants producing established embryogenic tissue were recorded.

Experiment 2: SE initiation on two nutrient media with an optimized PGR concentration. This experiment was conducted at CFS, Atlantic Forestry Centre (AFC), Fredericton, New Brunswick, Canada, with all 14 OP families collected at six dates (from June 28 to August 2).

Two SE nutrient media were used including the MLV medium described above, and a modified MLV, designated as AFC medium. For both media, the PGR concentrations were the same as in L-PGR medium. For the AFC medium, the ammonium nitrate level was reduced to 1/10, $MgSO_4$ was reduced to 1/3, while calcium was supplied as $Ca(H_2PO_4)_2$ at 200 mg l^{-1} instead of as $CaCl_2$ at 11 mg l^{-1} thus also changing the phosphate level in the medium.

Ten megagametophytes were cultured in each Petri dish containing approximately 25 ml of initiation medium. Depending on the availability of seeds, 60–100 explants were cultured per family, collection date, and medium. Thus 6–10 replicates (Petri dishes) per each parameter were used in the subsequent statistical analysis. Culture conditions were similar to those described in the previous experiment. Data were collected on number of explants producing established embryogenic tissue.

Maturation of somatic embryos and conversion to plants. From Experiment 1, 9–12 embryogenic tissue lines from each of the five OP families and per medium (S-PGR, L-PGR, and UL-PGR) were randomly selected (152 embryogenic lines), bulked, and used for maturation experiments. The ages of the cultures ranged from 3 to 6 mo. from the onset of initiation. Bulking and somatic embryo maturation protocols were the same as previously described (Klimaszewska and Smith, 1997; Klimaszewska et al., 2000), but with some modifications. The modifications included dispersing 200 mg fresh mass (fm) (instead of 300 mg fm) of embryogenic tissue over the filter paper placed on approximately 25 ml maturation medium in a 100 × 15 mm Petri dish. The MLV maturation medium contained 60 g l^{-1} sucrose (instead of 30 g l^{-1}) and 120 μ*M* abscisic acid (ABA, racemic, Sigma, instead of 80 μ*M*) and 10 g l^{-1} gellan gum. The aliquot of ABA stock solution was filter-sterilized and added to the molten autoclaved medium. Three to five Petri dishes (replicates) were used for each embryogenic line. Cultures were kept at approximately 24°C, low light intensity (5 μmol m^{-2} s^{-1}, cool white, fluorescent lamps GE F72T12/CW), and a 16-h photoperiod for 12 wk without subculturing onto fresh medium. The number of mature, phenotypically normal somatic embryos (having distinct cotyledons, embryonal root caps and smooth hypocotyls) was recorded.

Embryos were placed on the germination medium without any post-maturation treatment. The MLV medium included 20 g l^{-1} sucrose, 6 g l^{-1}

gellan gum, and no PGR. Thirty to 40 somatic embryos were placed horizontally on the germination medium in a 100 × 15 mm Petri dish with the cotyledons facing in one direction. Depending on the number of somatic embryos available, approximately, 30–200 somatic embryos were germinated per family per initiation medium. The Petri dishes were tilted to an almost vertical position with embryonal root caps pointing downward. For the first 2 wk, the growth proceeded at approximately 24°C and 16-h photoperiod with a low light intensity as described previously. Thereafter, cultures were placed at a higher light intensity (20 μmol m^{-2} s^{-1}) for 1 wk, and afterward the light intensity was increased to 40 μmol m^{-2} s^{-1}.

After 4–6 wk, germinating seedlings were subcultured onto fresh medium and their number per Petri dish was reduced to 8–12, depending on their size. After 10–12 wk, the number of plants was scored, and they were transferred into 1:1 peat:perlite mix in the greenhouse at CFS-AFC in May 2000. A high relative humidity was maintained with frequent misting irrigation, and after about 60 d, the plants were moved to regular greenhouse conditions. The light conditions were a 20-h period provided by high-pressure sodium illumination and the day/night temperature was 25/18°C.

Statistical models and analysis.. The frequency of SE in the two initiation experiments, expressed as a percentage, was subjected to analysis of variance using the model:

$$Y_{ijkl} = \mu + D_i + M_j + F_k + DM_{ij} + FD_{ik} + FM_{jk} + e_{ijkl},$$

where Y_{ijkl} is the percentage of lth plate of the ith collection date cultured in the jth medium for kth family; μ is the experimental mean; D_i is the effect of the ith collection date; M_j is the effect of the jth PGR level or initiation medium; F_k is the effect of the kth family; DM_{ij} is the effect of interaction between the ith collection date and the jth medium; FD_{ik} is the effect of interaction between the ith collection date and the kth family; FM_{jk} is the effect of interaction between the jth PGR level or medium and the kth family; and e_{ijkl} is the random error component.

The family (F_k) term was considered as a random effect, while all other main effects were considered as fixed effects. The percentage and arcsine transformed data were evaluated for normality with the Univariate procedure prior to analyses (SAS Institute). Since non-transformed data indicated a better normality, analyses were performed on actual percentage values. Variance components were estimated for the random effects, and means were calculated for fixed effects using the GLM procedure (SAS Institute).

Similarly, the number of mature somatic embryos per g fm of embryogenic tissue was subjected to analysis of variance using the model:

$$Y_{ijk} = \mu + P_i + F_j + PF_{ij} + e_{ijk},$$

where Y_{ijk} is the number of mature somatic embryos from the kth plate of the jth family initiated from the ith PGR level; P_i is the effect of the ith PGR level; F_j is the effect of the jth family; PF_{ij} is the interaction effect of ith PGR level and the jth family; and e_{ijk} is the random error component. Analysis of variance was performed on square root transformed data, but means were presented of non-transformed data.

Results

Seeds collected at the first date (June 28) contained translucent megagametophytes enclosing embryos at the pre-cleavage stage (Table 1). Subsequent collections included embryos at the post-cleavage stage through the stage of established dominance and finally precotyledonary and cotyledonary stages. Of the five OP families, one was initially more advanced in development, but by the fourth collection, no differences were noted.

Embryogenic tissue of *P. strobus* began to grow from the explants after the second week of culture with the majority of explants responding by the 12th wk. Embryogenic tissue could be initiated as late as after the 20th wk of culture.

For Experiment 1, analysis of variance indicated significant differences among collection dates, PGR concentrations, and collection date × PGR concentration interactions (Table 2). The variance component due to families was also significant, but variances due to family × collection date interaction and family × PGR concentration interaction were negligible. There were large differences among PGR levels for the initiation of embryogenic tissue (Fig. 1). Over all five families and collection dates, the L-PGR medium produced the highest initiation response with a mean of 35.0% (standard error, s.e. = 1.6), followed by UL-PGR with 28.9% (s.e. = 1.5) and S-PGR with 16.7% (s.e. = 1.2). The average initiation rate of SE was the highest in the July 5 collection at 38.0% (s.e. = 4.6) and declined to 13.4% (s.e. = 1.2) by the August 2 collection. For all collection dates, L-PGR medium consistently produced higher initiation rates ranging from 17.4 to 48.9%, while S-PGR produced the lowest response ranging from 10.1 to 21.2%.

TABLE 2

ANALYSIS OF VARIANCE FOR PERCENTAGE SE INITIATION IN *PINUS STROBUS* IN EXPERIMENT 1

Source of variation	df	Mean square	*F* value	Significance
Collection dates (D)	4	11 236.17	32.73	$P < 0.0001$
PGR levels (P)	2	14 941.70	43.53	$P < 0.0001$
Families (F)	4	1 893.83	5.52	$P < 0.0002$
D × P	8	1 491.54	4.35	$P < 0.0001$
F × D	12	529.81	1.54	ns
F × P	8	241.13	0.70	ns
Error	614	343.25		

ns, not significant at $P = 0.05$.

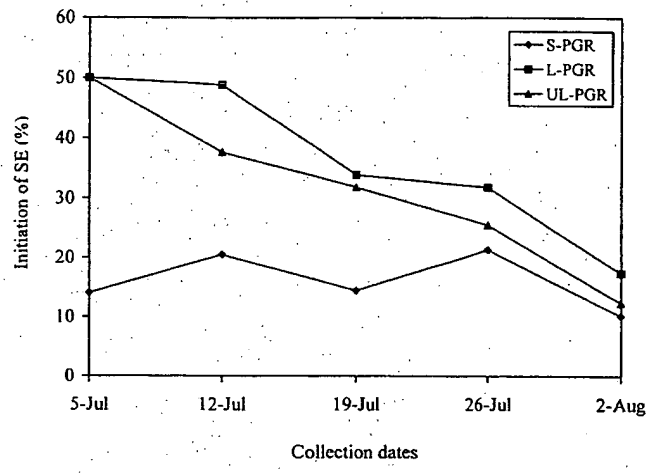

FIG. 1. Initiation of SE from five OP families of *Pinus strobus* on MLV medium with varying PGR concentrations over five collection dates in Experiment 1. Each point represents a mean of the five families for each PGR concentration.

The results from Experiment 2 were similar to those from Experiment 1 (Table 3). Collection dates had a major impact on initiation of SE. The initiation response on the first collection date (June 28) was the lowest with an average of 6.3% (s.e. = 1.0); however, 1 wk later (July 5), the SE initiation increased more than eightfold to the highest with an average of 52.9% (s.e. = 1.9) (Fig. 2). On subsequent collection dates, the initiation frequency declined steadily to 8.1% (s.e. = 0.6) by the August 2 collection. There was a significant difference between the two nutrient media tested, the percentage of SE initiation, averaged over all collection dates, was higher on MLV medium (34.1%, s.e. = 1.1) than on AFC

TABLE 3

ANALYSIS OF VARIANCE FOR PERCENTAGE OF SE INITIATION IN *PINUS STROBUS* IN EXPERIMENT 2

Source of variation	df	Mean square	*F* value	Significance
Collection dates (D)	5	56 575.92	81.79	$P < 0.001$
Media (M)	1	5 949.75	9.07	$P \leq 0.01$
Families (F)	13	2 793.76	3.40	$P < 0.001$
D × M	5	1 910.03	8.38	$P < 0.001$
F × D	64	691.70	3.04	$P < 0.001$
F × M	13	655.43	2.88	$P < 0.001$
Error	1022	227.85		

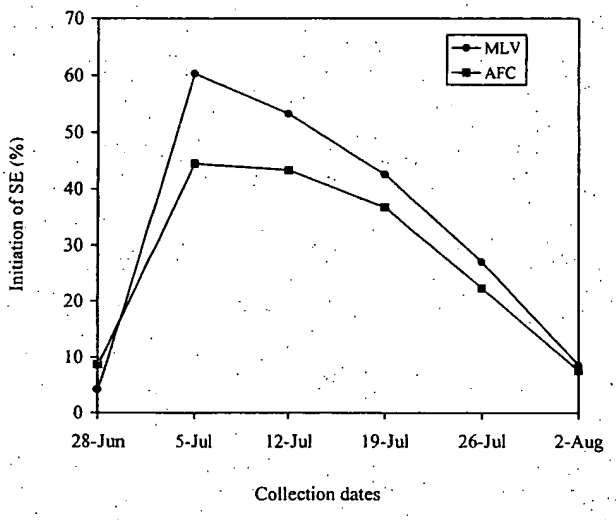

FIG. 2. Initiation of SE in *Pinus strobus* on MLV and AFC media at low PGR concentrations over six collection dates. Each point represents a mean of 14 OP families for each medium in Experiment 2.

TABLE 4

ESTIMATED VARIANCE COMPONENTS AND THEIR PERCENTAGES TO TOTAL VARIANCE IN EXPERIMENT 2

Variance component due to:	Estimate (SD)	%
Families (σ^2_F)	29.34 (15.34)	8.49
Family × collection date (σ^2_{FD})	35.63 (9.28)	10.31
Family × media (σ^2_{FM})	52.83 (29.59)	15.28
Error (σ^2_e)	227.85 (10.07)	65.92
Total (σ^2_T)	345.65	100.00

medium (28.3%, s.e. = 1.0). The SE initiation on AFC medium was higher than on MLV medium (8.7 vs. 4.2%), but only on the first collection date.

The relative magnitude of the variance component due to families (σ^2_F) amounted to 8.49% of the total genetic variance, while variances due to interactions of families with either collection date (σ^2_{FD}) or media (σ^2_{FM}) amounted to 10.31 and 15.28%, respectively, of the total variance (Table 4). However, the majority of variances was accounted for by genetic–environmental variances (σ^2_e). Although the genetic variance expressed by σ^2_F was smaller than

TABLE 5

ANALYSIS OF VARIANCE FOR NUMBER OF MATURE SOMATIC EMBRYOS OF *PINUS STROBUS* PER g FRESH MASS OF EMBRYOGENIC TISSUE IN EXPERIMENT 1

Source of variation	df	Mean square	*F* value	Significance
PGR levels (P)	2	118.31	3.60	$P < 0.03$
Families (F)	4	58.17	1.77	ns
P × F	8	28.21	0.86	ns
Error	137	32.86		

ns, not significant at $P = 0.05$.

TABLE 6

MEAN NUMBER OF MATURE SOMATIC EMBRYOS OF *PINUS STROBUS* PER g FRESH MASS OF EMBRYOGENIC TISSUE BY FAMILIES AND THE CONCENTRATION OF PGR IN THE INITIATION MEDIUM

	Concentration of PGR in the initiation medium			
Families	Standard (S-PGR)	Low (L-PGR)	Ultra-low (UL-PGR)	Mean (s.e.)
1053	113.5	159.5	172.3	147.0 (26.5)
1054	41.6	87.5	181.2	105.5 (22.8)
1055	86.1	178.3	123.7	132.5 (27.6)
1073	92.9	108.2	103.1	101.3 (20.3)
1148	39.5	76.8	90.2	68.2 (13.5)
Mean (s.e.)	75.8 (14.8)	122.6 (18.9)	134.7 (18.5)	

s.e., standard error.

the other genetic–environmental variances, it was still statistically significant. The family means of SE initiation frequency, combined over MLV and AFC media, ranged from 23.8 (family 1073) to 43.5% (family 1061). At the best collection date (July 5), explants of family 1061 produced embryogenic tissue at 81.4% while family 1057 produced it at 26.7%. Such wide-ranging differences among families at a given collection date contributed to significant interactions between families and collection dates (Tables 3 and 4). The general trend of the SE response showed a peak at the second collection date followed by a gradual decline in subsequent collection dates (Fig. 2). However, a few families continued to show an increase in the SE response from collection dates 2 to 3. This increase also contributed to a significant interaction variance. Mature somatic embryos were obtained from 145 embryogenic lines (of 152 lines tested) representing the five families used in Experiment 1. Analysis of variance indicated that there were significant effects among PGR levels for the number of somatic embryos produced, but the variance due to families and the interaction between PGR levels and families were not significant (Table 5). The S-PGR medium consistently produced a low number of mature somatic embryos (mean = 75.8) per g fm, while L-PGR and UL-PGR produced an average of 122.6 and 134.7, respectively (Table 6). The average production of mature somatic embryos by families ranged from 68.2 to 147.0.

Conversion of somatic embryos to plants occurred within 6–10 wk of culture at frequencies ranging from 70 to 82% (Table 7). Placing somatic embryos horizontally on the surface of the medium seemed to promote germination and plant growth (Fig. 3A–C). This

TABLE 7

CONVERSION OF SOMATIC EMBRYOS OF *PINUS STROBUS* TO PLANTS

Family (no. of lines)	No. of somatic embryos cultured	No. of plants produced	Conversion (%)
1053 (26)	1668	1374	82
1054 (19)	782	588	75
1055 (13)	1346	959	71
1073 (19)	1492	1178	79
1148 (16)	1024	715	70
Total (93)	6312	4814	76

position prevented the roots from penetrating the medium and promoted both root and epicotyl growth. When the roots penetrated the gelled medium, they often ceased to grow and eventually died (preliminary data not shown). About 5000 plants from 93 embryogenic lines representing five families were regenerated from somatic embryos (Fig. 3D), and a subset of these plants will be planted in the field.

Discussion

The concentration of PGR in the MLV medium had a significant effect on SE initiation rates in *Pinus strobus*. Exposure to medium with a lower than 'standard' PGR concentration resulted in significantly increased SE initiation. The L-PGR medium gave the highest consistent initiation rate for all collection dates. Although explants on the UL-PGR medium responded at a lower frequency than on L-PGR, the difference between the two media was small. These results indicated that the PGR concentration in L-PGR medium was optimal for the initiation of SE in *P. strobus*.

Plant growth regulator concentrations in the SE initiation medium have not been extensively studied to date. However, in *P. taeda*, modifying the level of growth regulators for initiation has also yielded positive results (Li et al., 1998), although the 2,4-D concentration was much higher than reported in this study or for *P. monticola* (Percy et al., 2000). This difference in the requirement for exogenous PGR may be due to interspecies variation in the endogenous hormonal environment within the developing ovule, and may affect the exogenous culture requirement (Banerjee and Radforth, 1969).

Previous attempts to induce SE on media lacking PGR produced varying rates of success in different pine species (Becwar et al., 1990; Nagmani et al., 1993; Jones and van Staden, 1995; Lelu et al., 1999). An attempt to initiate SE in *P. strobus* on a PGR-free MLV was unsuccessful, suggesting the indispensability of PGR at least in this medium (Klimaszewska, unpublished).

In this study, we have observed that SE initiation increased from 6.3% with the June 28 collection to 52.9% with the July 5 collection. Based on the visual assessment of developmental stages of the zygotic embryo, we have observed pre-cleavage stage embryos in the July 5 collection, thus most fertilizations probably occurred between June 28 and July 5. This indicated that most SE in *P. strobus* was initiated prior to the formation of distinct subordinate embryos. Subordinate embryos are formed when cells of a four-celled proembryo separate and individually form a new embryo (i.e., cleavage polyembryony). Of these four embryos, one generally becomes dominant and continues to grow while the others remain small and eventually degenerate (Dogra, 1978). We speculate that most of our embryogenic tissue may have resulted from auxin-induced cleavage of the four-celled proembryo followed by further cleavage later on. An embryogenic system similar to that described for other conifers (von Aderkas et al., 1991; Filonova et al., 2000) presumably ensues immediately following the first cleavage or thereafter.

In our *P. strobus* cultures, maximum initiation occurred at the four-celled proembryo stage, but some initiation was still achieved in explants from seeds derived from cones collected as late as August 2. At that stage, the dominant embryo was cotyledonary and some subordinate embryos were still present. We do not know whether initiation at this late stage of development is from the dominant embryo or from the subordinate embryos; we presume that it occurs from both but probably not equally. In *P. sylvestris*, a similar pattern of response was described except that SE initiation did not necessarily require the presence of PGR in the medium (Lelu et al., 1999).

The mineral composition of the medium was shown to affect SE (Preece et al., 1989; Gyulai et al., 1992). Litvay (LV) medium is high in ammonium. However, high ammonium levels had a negative impact on SE and organogenesis in several species, including some conifers (Verhagen and Wann, 1989; Tuskan et al., 1990). Therefore, in AFC medium the ammonium nitrate level was greatly reduced. LV medium, in comparison to other media, is also high in magnesium and very low in calcium. In AFC medium the levels of these components were adjusted to a level more common in tissue culture media. High potassium phosphate levels have inhibited ethylene biosynthesis (Chalutz et al., 1980) and stimulated shoot formation (Miller and Murashige, 1976) in some plants. In shoot explant cultures of *P. caribaea* the number of adventitious shoots that formed and the percentage of foliage that was free of necrosis correlated positively with the amount of phosphorus consumed by the explant. Therefore, the phosphate level was doubled over that in LV medium. However, these alterations in mineral content did not result in a medium better suited for SE initiation than the MLV medium. As in Experiment 1, the SE initiation rate on MLV and AFC media was the highest when megagametophytes were cultured with embryos at the pre-cleavage stage or immediately thereafter, i.e., before dominance was established. The combined results from the two experiments indicated that the critical factors in *P. strobus* SE initiation included the developmental stage of zygotic embryos and the PGR concentration in the culture medium, whereas the nutrient composition of the medium appeared to be less critical.

The genetic influence in initiation of SE was indicated by the variance component due to families (σ^2_F), which amounted to 8.49% of the total variation. This variance component represented the covariance of half-sib (OP) families and, as such, can be interpreted as one-quarter of the additive genetic variance. However, due to potential mixture of full-sibs within the family, the variance component may be closer to one-third of the additive genetic variance (Squillace, 1974). Based on this assumption, the estimated heritability for SE initiation (h^2) defined as the ratio of additive variance to total phenotypic variance was 0.25. This estimate is considered to be large when compared with other quantitative traits in forest trees and indicates that selection of responsive families can be done effectively. The interactions of

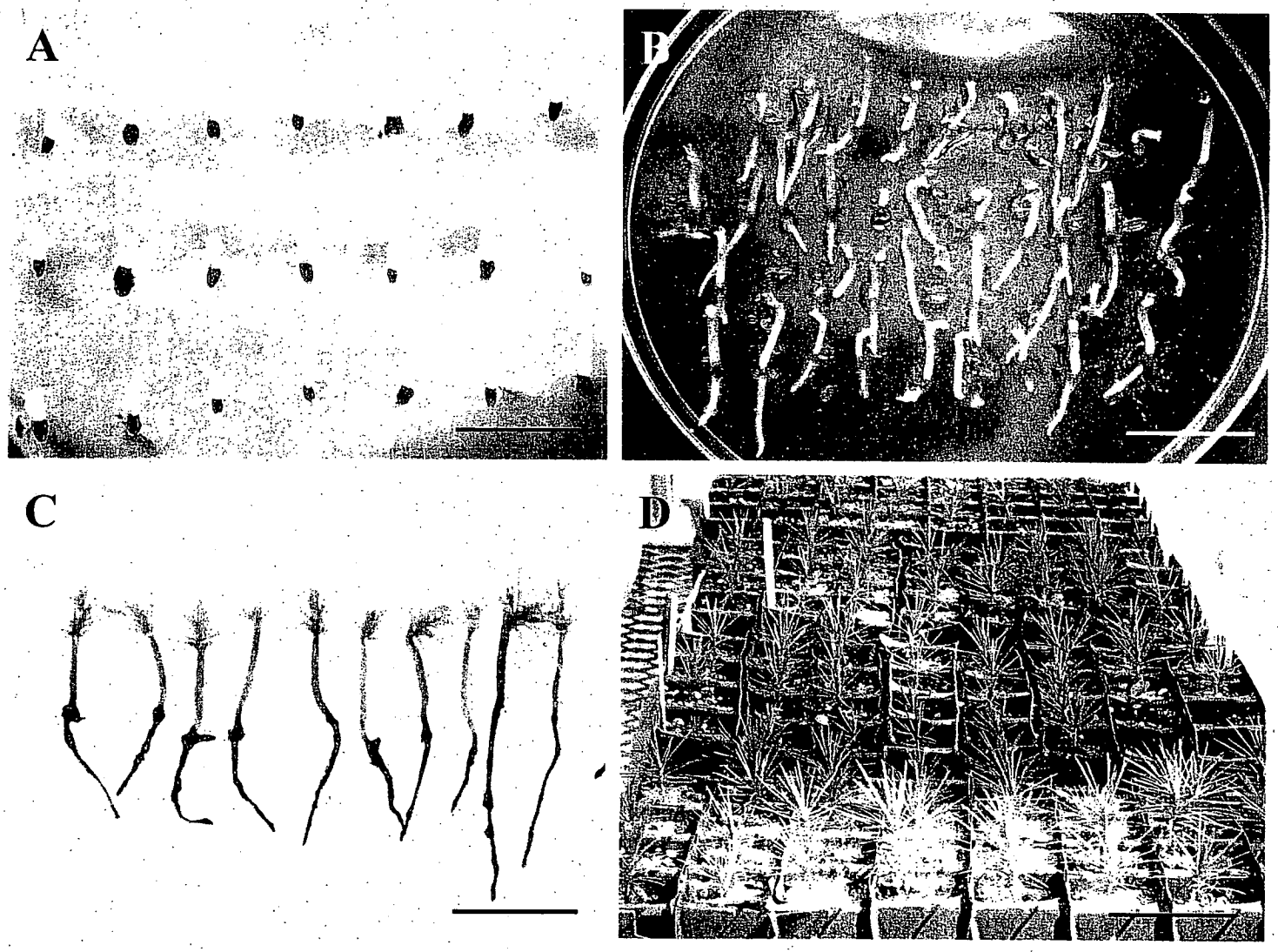

FIG. 3. Conversion of somatic embryos of *Pinus strobus* to plants. A, Somatic embryos after 2 d on the germination medium (*bar* = 1 cm). B, Somatic embryo-derived seedlings after 2 wk of growth on the germination medium (*bar* = 2 cm). C. Somatic seedlings after 10 wk of growth on the germination medium and at the time of transplanting to a greenhouse (*bar* = 2 cm). D, Somatic seedlings after 8 wk of growth in a greenhouse (*bar* = 5.3 cm).

collection dates and media with families accounted for even larger amounts of variance, reaching about 26% of the total variance.

Our results also indicated that PGR in the culture medium prior to maturation had a significant effect on the production of mature somatic embryos. Embryogenic tissue from MLV S-PGR, which had the highest concentration of 2,4-D and BA, consistently produced a low number of mature somatic embryos for all five OP families tested. The auxin 2,4-D accumulated to a higher level in plant tissues and was less rapidly conjugated (inactivated) than other auxins (Ribnicky et al., 1996). Thus there is a possibility that residual amounts of the PGR persisted in the embryogenic tissue of *P. strobus* and that this might have inhibited the development of some somatic embryos on the maturation medium.

In conclusion, a high frequency of SE initiation in *P. strobus* was achieved by manipulating PGR concentrations in the culture medium. The best developmental stage of zygotic embryos for SE initiation was the pre- to shortly post-cleavage stage. The optimized initiation medium was MLV with low PGR (L-PGR). Furthermore, the embryogenic tissue initiated and maintained on this medium consistently produced a higher number of mature somatic embryos relative to the same medium with S-PGR. There were no differences in yield of mature somatic embryos from embryogenic tissue derived from the L-PGR and UL-PGR media. As expected, there was a substantial genetic influence on initiation of SE as indicated by a significant variance component due to families and their interactions with PGR levels and the developmental stages of zygotic embryos. Although it was variable among families, SE was obtained for all the families at all collection dates.

The high rate of SE initiation and improved somatic embryo maturation protocol indicate that a commercial application of *P. strobus* in clonal forestry is feasible. Integration of SE in *P. strobus* breeding programs and large-scale propagation of selected genotypes, showing increased tolerance to the pathogens such as white pine blister rust and white pine weevil, would potentially make this species more attractive for commercial plantation forestry.

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